Science within Food: Up-to-date Advances on Research and Educational Ideas

FORMATEX
Food Science Series N° 1

Edited by

A. Méndez-Vilas

ISBN-13 Collection: 978-84-947512-1-9

Formatex Research Center
C/ Zurbarán, 1, 2° - Oficina 1
06002 Badajoz
Spain
http://www.formatex.org

Printed in Spain

TABLE OF CONTENTS

Introduction to the book series

Dear Reader,

The present edition is the first number of the Food Science book series, which belongs to a more general line of books published by Formatex, aimed at disseminating current scientific and technological research in a way accessible to a public beyond the very and increasingly specialized academic one.

In the case of this Food Science series, it serves to give an overview of the state of the art, as well as upcoming trends, and to promoting discussion about scientific, technological and educational aspects related to food science.

We called for pieces of work falling into two categories: 1) research papers prepared, as said above, in a not too specialized format (for example, introductory works showing the characteristics of the certain microscopy technique, or a certain aspect or application of it), and 2) mini-review papers, focused on either a certain important topic, or on the evolution of a certain research group across the years (that is, how and why certain research approaches were reinforced and other refused).

We hope the efforts put into the setting up of this number of the new series will be rewarded with extensive downloading of these articles from the edition website, and look forward to receiving new proposals for the new edition in 2019.

A. Méndez-Vilas
Editor

A Major Concern in Food Industry, Contamination Reservoir: Bacterial Biofilm

B. Uymaz Tezel[1] and **P. Şanlıbaba**[2]

[1] Food Technology Program, Bayramiç Vocational School, Çanakkale Onsekiz Mart University, Çanakkale, Turkey
[2] Department of Food Engineering, Engineering Faculty, Ankara University, Gölbaşı, Ankara, Turkey

To resistance to inhospitable environmental stresses bacteria have upgraded a variety of mechanisms such as biofilm formation. This self-protection growth pattern of bacteria makes them more resistant to disinfectants and antimicrobial agents since it provides a barrier which prevents or lessens the contact with antimicrobial agents. Formation of biofilms, which acts as a contamination reservoir, is now regarded as a major concern in the food industry since biofilms may contain potential spoilage and pathogenic bacteria including *Listeria monocytogenes*, *Staphylococcus* spp., *Lactobacillus* spp., *Clostridium* spp., *Bacillus* spp., *Salmonella* spp., *Escherichia coli* O157: H7 and *Campylobacter jejuni* etc. Many factors can be sorted for the formation and development of biofilms: specific bacteria strain, material surface properties and environmental parameters such as pH, temperature and nutrient levels. Each biofilm is different due to the diversity of biofilm-forming bacteria in various food industries, as a result, there is no unique system which can remove all biofilms. The first and perhaps the most important thing to do is prevent biofilm formation by regularly cleaning and disinfecting and biofilm problem should be well-analysed to determine an effective cleaning and disinfection operation.

Keywords: Food industry; bacterial biofilm; contamination reservoir

1. Introduction

Foodborne diseases are widespread and crucial public health threat which are the cause of hospitalisation, mortality or significant impediment to socio-economic development. According to global estimates, thirty-one foodborne hazards causing 32 diseases most of which are diarrhoeal disease agents particularly norovirus and *Campylobacter* spp. According to World Health Organization 2015 reports the 31 hazards caused 600 million foodborne illnesses and 420,000 deaths in 2010 however, unfortunately, the real number is likely to be much higher. Foodborne diarrhoeal disease agents caused 230,000 deaths most of whom are children [1]. The burden of foodborne diseases in industrialised countries is still an imported challenge. In a recent report (2105) by the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (EDC) a total of 5196 foodborne outbreaks were reported in the European Union (EU) in 2013 [2]. According to another statistics released by the United States Centre for Disease Control and Prevention (CDC) emphasise that conjecturally, 48 million foodborne diseases reported resulting in 128,000 hospitalisations and 3000 deaths annually in the USA, with estimated money loss of billions dollar in a single year [3]. Microbial contamination, both from microbiological or chemical, may occur at different steps of the food chain: (1) cultivation environment such as farm, orchard, fishery or livestock farms (2) processing environment such as slaughterhouse, cannery or packing plants (3) preparation, storage and even retail environments [4]. Members of EU have adopted an approach to food safety from the farm to the fork for the protection of consumers against microbial contamination throughout the food chain [5, 6]. Knowing main risks and contaminants in each food processing steps is crucial for their control, prevention or elimination. In order to manage the production of safe and quality foods adoption of risk assessment models such as Hazard Analysis Critical Control Points (HACCP) and Good Manufacturing Practices (GMP) are essential, even though these systems have proven very effective for the control of food safety, it must never be realized that they are designed on the basis of known hazards. Both human and microorganisms related factors lead to evaluation of food safety which occurs on a molecular scale (point mutations or the acquisition of a plasmid by microorganisms) and on short timescales. Innovations according to new validations and verifications are necessary [6]. Furthermore, many bacteria have upgraded a variety of mechanisms such as biofilm formation to be resistant to inhospitable environmental stresses. Formation of biofilms, which acts as a contamination reservoir, is now regarded as a major concern in the food industry since biofilms may contain potential spoilage and pathogenic bacteria including *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* O157: H7, *Campylobacter jejuni*, *Bacillus* spp., *Pseudomonas* spp., even *Lactobacillus curvatus*, *Lactobacillus fermentum* etc. [7]. The biofilms increase the risk for post-processing contamination and leading to lowered shelf life of the food product and/or transmission of diseases [8, 9, 10, 11]. Moreover, the formation of biofilms on industry surfaces leads to economic losses due to mechanical blockage, impairment of heat transfer, increase in fluid frictional resistance and corrosion of equipment [12, 13].

In order to gain deeper information about biofilms, a large of number research has been performed in recent years which are focused on both understanding of biofilm formation mechanisms and their effective and control strategies. The main objective of this book chapter is to emphasise the challenges caused by biofilms in food industries and to summarise the knowledge of biofilm formation of foodborne bacteria.

2. Bacterial Biofilm

Bacterial biofilms are defined by Costerton and Stewart [14] as an aggregation of microorganisms attached to and growing on surfaces including plastic, glass, metal and wood which are a prevalent mode of growth for microorganisms in nature [15]. The current description of bacterial biofilms is performed as an assemblage of surface-associated microbial cells that are enclosed in hydrated (from 85% to 95%) extracellular polymeric substances (EPS) mainly constituted of polysaccharides, proteins, phospholipids, teichoic and even nucleic acids [16, 17].

The first observation of bacterial biofilm dates back to 1684. Antonie van Leeuwenhoek, using his primitive light microscope, found that microorganism attached to tooth surfaces, form a sessile community and called them as 'animakuli' [15, 17, 18]. According to the findings of light microscopy bacterial biofilm was observed first as marine bacteria attached to the ships' hulls in the 1920s [15]; Claude E. ZoBell also determined that some marine bacteria could cling to surface of glass slides, form microcolonies and also these structures on the glass surfaces was not distributed by washing or shaking [19]. In the 1980s, by the development of the microscopy techniques, bacteria were observed on the solid surfaces of many ecological environments including wastewater, treatment systems, equipment used to manufacture vinegar, industrial water systems, tooth decay, urinary tract and other implanted medical devices [20, 15]. The first correct question to ask is why bacteria create a structure as a biofilm. This proper colonisation of microorganisms on surfaces, embedded in the EPS, provides survival of bacteria under environmental stress conditions such as ultraviolet radiation, physicochemical stresses or insufficient supply of nutritive resources. This self-protection growth pattern also provides bacteria up 10 to 1000 times more resistance to disinfectants and antimicrobial agents than planktonic bacterial cells [21, 22, 23]. The biofilm structure acts as a barrier and prevents or decreases contact of the bacteria with antimicrobial agents. In order to make pathogens inactive and maintain safe and quality products, chemical disinfectants such as peroxides, chloramines or hypochlorites are widely used in food industry. However, it has been reported in the United States that around 80% of persistent bacterial infections are associated with biofilms [24].

2.1 Biofilm formation and development steps

To explain this threatening issue in a broad range of food industries including brewing, seafood processing, dairy processing, poultry processing and meat processing the second question to ask is which molecular mechanisms, factors and bacteria are involved in biofilm formation.

The biofilm formation process that involves both physicochemical and biological factors starts with the initial attachment of planktonic bacteria to solid surfaces and takes in a short time and it can be active (depend on bacterial motility) or passive (gravitational transportation of planktonic cells) [17, 24, 25, 26]. The attached microorganisms may detach from the surface and return to planktonic forms at any moment, it is considered as a reversible adhesion of the bacteria and surface through van der Walls, electrostatic and Lewis acid-base interactions. The main determinant factor is physiochemical properties of the bacterial surface – mostly negative charged- due to the presence of the proteins anchored to the cell wall [25]. For example, the electrostatic charge of bacterial cell walls and cell surface hydrophobicity of *L. monocytogenes* have been the determinate that govern its attachment to stainless steel [27], also BapA surface protein has been found to be directly involved in biofilm formation at the liquid-air interface in *S. enterica* [28]. The cell surface appendages such as flagella (present in both Gram-positive and Gram-negative bacteria), pili (thinner and shorter than flagella, present in both Gram-positive and Gram-negative bacteria) or curli fibres (amyloid like protein, present in only Gram-negative bacteria) not only the principal components for bacterial motility, but also may play a crucial role (involved in cell-cell and cell-surface contacts) in the initial stages of biofilm formation [29, 25].

The other important factors that influence the initial cell attachment are the physical characteristics of solid surfaces such as texture (rough or smooth), surface charge, hydrophobicity and weather be covered by a film of organic molecules (proteins or pre-existing polymeric substances). For instance, high free energy, wet surfaces promote bacterial adhesion: Although hydrophilic surfaces (stainless steel, glass etc.) are more adherent than hydrophobic surfaces (plastics) [17, 24, 30], rougher and hydrophobic surfaces are preferred attachment. According to findings of research by Sinde and Carballo which shown that *Salmonella* and *Listeria* can attach in high numbers to hydrophobic surfaces than the hydrophilic ones [31].

The production of EPS induces to turn weak interaction between bacteria and surface to a permanent bonding (by dipole-dipole, hydrophobic, ion-ion, ion-dipole, covalent and hydrogen interactions); so this stage of biofilm formation is called as an irreversible attachment. In the irreversible stage the biofilm grows through cell division and bacterial cells upregulate the expressions of specific attachment-related genes [17, 23, 24, 26]. The microcolony formation in the early development of biofilm architecture stage helps strengthen the bond between the bacteria and the substratum and stabilises the colony from any environmental stress and also allows for interspecies substrate exchange [32].

The maturation stage usually has been characterised by developing of flat or mushroom shape which requires periods of 10 days or more [17, 24, 26]. A mature biofilm is a highly organised ecosystem including heterogeneous complex-enclosed microcolonies with water channels are dispersed and able to provide passages for the exchange of nutrients, metabolites and waste products [33].

The third question to ask is how different bacteria microcolonies have attended to biofilm architecture. Although its role has not been completely explained, Quorum Sensing (QS) which is a kind of communication between bacteria responsible for heterogeneous biofilm architecture and provides information about cell density for adjusting their gene expression [23]. QS includes production and sensing of signal molecules which regulate the behaviour and physiological processes of bacteria such as growth, sporulation, pathogenicity or starting to biofilm formation [34, 35, 36]. There have been several studies emphasise effect of the QS molecules in different bacteria [25]: the second messenger cyclic-di-GMP responsible from cellular transition between motility and sessility in *S. enterica* and many other pathogens [37, 38]; the accessory gene regulator (*agr*) system plays a central role to mature biofilm phenotypes and 3D architectures in *S. aureus* [39, 40]; the CodY regulator, induces both biofilm formation (under nutrient starvation conditions) and expression of motility and virulence factors (under abundant nutrient conditions), controls the multicellular behaviour in *B. cereus* [41].

The detachment of bacterial cells from biofilm architecture and revert to their planktonic form is the last stage in the biofilm formation cycle, called as Dispersion. Both external and internal biofilm processes may lead to biofilm detachment [24]. The dispersion stage is an active process which has been controlled by QS signals and allows for the colonisation of new niches. Generally, the acly-homoserine lactones (AHLs) and oligopeptides are the extracellular signalling molecules for Gram-negative and Gram-positive bacteria, respectively [42]. The *agr* system also controls dispersion in *S. aureus* biofilm by the activation of protease production [43]. Sometimes some molecules produced by biofilm cells disrupts other species' biofilm architecture. For example, the polyamines as norspermidine and norspermine produced by *B. subtilis*, disrupt the *S. aureus* and *E. coli* biofilm architecture [44].

Many effective factors can be sorted on the formation and development of biofilms: specific factors of bacterial strain (Gram-positive/negative, microbial shape, structure, presence of flagella, pili, capsules or exopolymeric substances molecular composition, species, growth phase and age), material surface properties (chemistry, topography, physiochemistry) and environmental parameters such as pH, temperature, nutrient levels and fluid dynamics [15, 17, 23]. The factors grouped as environmental parameters may be considered to play a crucial role in the turning of planktonic cells to the sessile form. There are several evidence demonstrate that the different adhesion levels of various species at different pH, temperature and concentrations of phosphates or pH. Main effects of environmental parameters on biofilm formation in the food industry can be summarized as follows: rough, hydrophobic and opposite charges of surface favours biofilm initiation; lower temperatures either stimulate biofilm formation which leads to more uniform properties of polysaccharides- or lower biofilm formation -decrease cell surface hydrophobicity levels; decrease of oxygen inhibits biofilm initiation; higher shear rates decrease bacterial attachment but increase density and thinness of biofilms; high osmolarity of food matrix inhibits biofilm formation; influence of pH and ionic strength on biofilm formation through changes in surface hydrophobicity and charge [45]. In addition to abiotic environmental factors listed above, one should consider that the effect of biotic factors is also decisive. Interactions between different microbial populations, especially in multi species biofilms, could greatly impact the development of biofilm as far as the species specific factors. Whereas these interactions mostly are beneficial for the partners, in some cases presence of a bacterial species could inhibit biofilm formation. For example, while *L. monocytogenes* in a mixed biofilm with *Lb. plantarum* exhibited higher resistance to benzalkonium chloride and peracetic acid than single species biofilm [46]; biofilm formation of *L. monocytogenes* can be reduced by the presence of *S. xylosus* and *P. fragi* or bacteriocin-producing *Lactococcus lactis* [29, 45, 47].

3. Biofilm formation in Gram-positive and negative bacteria

According to the nature of microorganisms and depending on the species-specific factors biofilms have displayed diversity. Each biofilm is different due to the diversity of biofilm-forming bacteria in different food industries and also same species can form different biofilm architecture under different grown conditions. According to evidence of SEM, epifluorescence microscopy and confocal laser scanning microscopy (CLSM), biofilms of *L. monocytogenes* under static conditions consist of a homogeneous layer of cells with a similar morphology to planktonic cells but, under continuous flow conditions consist of spherically shaped microcolonies [7, 48]. The structural and developmental features of bacteria such as motility and sporulation are eminent importance for the biofilm formation and are strictly connected with each other. Formation of biofilms provides an optimal environment for sporulation of *Bacilli* spp. Although, cells in biofilm architecture are not motile, in several bacteria such as *L. monocytogenes* and *B. subtilis* motility has been found to be substantial for initial attachment of cells to a surface and formation of biofilm [7]. The flagellar based motility appeared to be essential to propel cells towards the surface before attachment for static biofilm formation but not for biofilm formation under continuous flow conditions in both *L. monocytogenes* and *B. subtilis* [49, 50]. In this situation evaluating biofilms according to the structural and developmental features of microorganisms, growing conditions and food processing environments is a rational approach.

3.1 Biofilm forming Gram-positive bacteria

L. monocytogenes is a psychotropic bacterium which can lead to mild gastroenteritis or severe infections and mostly transmitted to man through food. *L. monocytogenes* strains can grow between pH 4.6 and 9.5 and at 0.92 aw and are recurrently found on floors, drains and equipment of the food industry, even in the cold and wet atmosphere of refrigerated rooms where non-psychrotrophic can only survive. The studies focused on to understand why *L. monocytogenes* persists in the food industry have found several reasons including adhesion potential, biofilm forming ability, resistance to desiccation, acid and heat, tolerance or resistance to disinfectants[51]. It has been demonstrated that *L. monocytogenes* can form a biofilm which protects cells from the action of antimicrobials and sanitizers and allows long term persistence them on surfaces such as rubber, plastics, glass and stainless steel [52]. Having flagella, QS and extracellular DNA (eDNA) may play a major role in the formation of listerial biofilm. The attachment of *L. monocytogenes* has been governed by electrostatic charge of bacterial cell walls (conferred by peptidoglycan anionic teichoic acids) and cell surface hydrophobicity (enhanced by the presence of lactic acid) [17]. *PrfA* flagellar biosynthesis regulator, *LuxS* system, *agr* system have been shown to be involved in the QS systems of *L. monocytogenes* biofilm. Alonso et al., [53] found that both disruptions in several purine biosynthesis genes and mutation in flagella related genes at 30-35 ^{o}C caused reduced biofilm formation in *L. monocytogenes*. Piercey et al., [54] carried out that at a temperature common to food environments identified the involvement of nine genetic loci that had not previously linked to biofilm formation in *L. monocytogenes* as well as ten genes also known to be associated with *L. monocytogenes* biofilm temperatures. The role of eDNA has been described by Harmsen et al., [55] in the early stages of cell attachment to surfaces, and also reported that N-acetylglucosamine acted as a co-factor and/or scaffolding in eDNA formation.

As discussed above, the biofilm formation is strongly affected by the hydrophobic or hydrophilic interactions between microbial cell charge and contact surfaces. For the risk assessment and reduction of biofilm production which are a great expense for the enterprises, several measures should be taken as the choice of correct materials for installations. Both the hydrophilic (stainless steel and glass) and the hydrophobic (polymeric) materials are the most common materials used in the food industry [52]. According to several researchers who are investigating relationships between biofilms and contact surfaces suggest that *Listeria* biofilms may adhere more tightly to hydrophobic surfaces than hydrophilic surfaces at cellular level [56]; but some found that biofilm levels of *L. monocytogenes* were significantly higher on glass at different temperatures compared to polystyrene and stainless steel [57]. By the same researchers, a positive correlation between hydrophobicity and heat was suggested that the biofilm formation was influenced by temperature due to modification of cell surface hydrophobicity. The presence of glucose or high concentrations of NaCl (4% to 10%) may lead to an increased ability to self-aggregation and biofilm development in *L. monocytogenes* [58, 59]. With regard to a study conducted on the influence of growth medium, surface and pH on the initial 2D structure of static listerial biofilms, three of the tested strains were able to reach a honeycomb like structure and could develop complex biofilms but only one could not enter to the second step. Time-shift differences according to growth medium and surface have been observed on the plastic surface, except one strain. However, the first step of the primary structure of *L. monocytogenes* which is a critical stage for the development of listerial biofilms, is impaired for all strains in acidic conditions [60]. In accordance with this study, both Tresse et al., [61], and Smooth and Pirson [62] observed a lower cell attachment at pH five than pH 7 or 8. The down-regulation of flagellin synthesis in acidic conditions was also reported by Tresse et al., [63]. The dependency of *L. monocytogenes* biofilms on nutritional conditions is one of the most studied subjects. Although most of the previous studies show that growth in rich media (BHI or TSB) was not lead to switch cells from planktonic to sessile state [64], but growing in synthetic media enhanced attachment to surfaces and biofilm formation [65].

Due to the contamination of food products by both pathogens and spoilage microorganisms is a crucial issue the management of the food processing plant should consider the prevention strategies. The proper design of food contact equipment as the primary strategy should be performed. This should never be disregarded that the most common sources involved in biofilm accumulation are the floors, waste water pipes, bends in pipes, rubber seals, conveyor belts, etc. Materials for industrial installations should be preferred with smooth, resistant to corrosion and damage, be free of sharp edges, easily cleanable and allowing the 'clean in place' [17, 52].

Because of its rich nutrient content and high aw, milk is tremendously vulnerable to contamination against microorganisms. Outbreaks of pathogens associated with biofilms in the dairy industry are including *L. monocytogenes*, *Bacillus* spp., *Pseudomonas* spp., even *Lactobacillus curvatus*, *Lactobacillus fermentum* [17, 66, 67]. According to results of a study aimed to evaluate the biofilm production of *L. monocytogenes* strains isolated from the environment of cheese processing plants in Brazil, all isolates showed ability to produce biofilms on polystyrene microplates, also on stainless steel (24.7%) and 4.7% of the strains were classified as strong biofilm producer [68]. *L. monocytogenes* is able to form multi-species biofilms with both Gram-positive and negative bacteria depending on the genera implicated and the environmental conditions [69]. In a recent study which aimed to find out possible environmental relationships among *L. monocytogenes* and other species, environmental samples belonging to work surfaces from fish, meat and dairy industries were analysed by serotyping and PFGE. 12 samples were found positive for *L. monocytogenes* and *E.coli* and *Carnobacterium* spp. were founded accompanying microbiota in fish and meat industry, respectively [70]. Although the quality of both equipment and water is the main cause of the risks in the fish processing industry, many

types of fish-contaminated-bacteria are found to be biofilm-forming, including *Vibrio* spp., also many other genera such as *L. monocytogenes*, *Salmonella* spp., *Bacillus* spp., *Aeromonas*, and *Pseudomonas* spp., [17].

Mainly three conventional methods have been applied including physical, chemical and biological methods in order to prevent or inhibit the biofilms in food-processing plants. The physical methods commonly used are mechanical scrubbing, jet cleaning, ultrasonic cleaning and other ways [71]. The strong oxidising agents with a broad antimicrobial spectrum such as hypochlorous acid, chlorine, iodine, ozone, hydrogen peroxide, peroxyacetic acid and quaternary ammonium chloride and anionic acids are the most used chemical agents for sanitation procedures in food producing plants [52]. Several comparative experimental studies have been performed in order to establish the most effective treatment for eradication *L. monocytogenes* biofilm. The gaseous ClO_2 [72]; aerosolised sanitizers [73] and nanostructured titanium dioxide combined [74] have been effective on both *L. monocytogenes* planktonic cells and biofilms. With the increasing consumer demand rather than chemicals preferring environment-friendly treatments launch to researching new emerging strategies. These innovative strategies can be sorted as affecting cell adhesion by modifying contact surface hydrophobicity [75], using different essential oils as anti-biofilms agents [76], using bio-solutions such as probiotic [77], bacteriocin-producing strains [78] or phages [79] and using enzymes, antimicrobial molecules from microbial origin [80]. In a study was conducted with a innovate approach, the methanol extracts of several plants have been found out to be effective on *L. monocytogenes* and *S. aureus* biofilm formation [80].

Staphylococcus spp. is widely distributed in nature including air, water, body surfaces of skin which is bunch shaped cocci without flagella, spore and capsule. The enterotoxin of *S. aureus* causes food poisoning which is a significant concern in the food industry. There for the primary enterotoxigenic pathogenic carriers are humans, food handlers may directly contaminate food. The presence of methicillin-resistance and ability to develop biofilms on different materials also makes a serious risk for safety food production [71]. According to a comparative investigation on biofilm formation capability of many common pathogenic bacteria indicated that *Staphylococcus* tend to develop more biofilm than *E.coli*, *Salmonella* spp., and *Bacillus cereus* [81]. Among the Staphylococcal species *S. aureus* and *S. epidermis* adhere to both biomaterials and food contact surfaces and form a biofilm in 24-48 hours at 37 ^{o}C. The process of heterogeneous multi-layered *S. aureus* biofilm formation is regulated by multiple genes including expression of the PIA by the *ica*ADBC operon; release of eDNA; expression of numerous surface proteins (Bap, SasG, FnBPs or Spa) [82]. eDNA has been reported as a major component of biofilm which has roles in initial attachment and accumulative stages of *Staphylococcus* spp. [83]. The PCR based analyses have shown that eDNA is similar to genomic DNA which supports the hypothesis that eDNA originates from the lysis of a subpopulation of the bacteria [84]. According to results of a study aimed to investigate the silico biofilm production ability of *S. aureus* from dairy environments, 31 *S. aureus* were obtained and detected as producer of biofilm on stainless steel, rubber and silicon surfaces [85]. Lopez et al., [86] reported that the glycone (myricitrin, hesperidin and phloridzin) and aglycone (myricetin, hesperetin and phloretin) flavonoids inhibit biofilm formation by *S. aureus* strains by overexpressing the *msrA* and *norA* efflux protein genes.

Lactic acid bacteria (LAB) are non-motile bacteria which have been widely used for the fermentation of raw milk, meat and vegetables. In spite of their role in the food industry, LAB -especially genus *Lactobacillus*- may lead to food spoilage. Non-starter *Lactobacillus* spp. can produce biofilm on the both raw material and production environment which acts as contamination reservoir [87]. Although many biofilm producer bacteria such as *Listeria* spp., *Bacillus* spp. and *Salmonella* spp. have flagella which are necessary for initial attachment to surfaces, the adhesion of non-motile bacteria to surfaces can be explained by the sedimentation processes, so the initiation of biofilm and maturation of non-motile bacteria is different from the reported for motile bacteria [79, 88]. There is another risk for the fermented foods and beverages that capacity of biogenic amine (BA) producers to form biofilms. The histamine, tyramine and putrescine are low-molecular-weight organic compounds that may found in large quantities. However much BAs play a major role in human physiology, ample consumption of food containing them may lead to toxicological effects. The aminoacyl decarboxylase activity which mainly presents in Gram-positive bacteria of the LAB group is responsible for the formation of BAs [89]. The formation of biofilms by both clinical and food-related *Enterococcus* spp. species have been studied since their pathogenic potential [90, 91].

Pseudomonas spp. includes major proteolytic enzyme producer species including *Pseudomonas aeruginosa* which is pathogenic and spoilage bacterium commonly found in food processing plants and has the tendency to form biofilms [92]. So much so that *Pseudomonas aeruginosa* has demonstrated biofilm formation in the lungs of patients suffering from cystic fibrosis which is noncurable and eventually results in death [93]. In order to inhibit biofilm formation, many studies have been focused on the AHLs QS system which is involved in biofilm formation by knock-out AHLs synthase genes [94]. Another biofilm producer Gram-positive bacteria is *Clostridium perfringes* which are aerotolerant anaerobic, spore-forming and an opportunistic pathogen. *Clostridium perfringes* causes food poisoning both in human and animals as result of its ability to produce many different toxins. The production mono- and dual- species biofilm protects *Clostridium perfringes* cells from the action of potassium monopersulfate, quaternary ammonium chloride, and hydrogen peroxide and glutaraldehyde solutions [95].

3.2 Biofilm forming Gram-negative bacteria

Salmonella spp. is one of the most important foodborne pathogens and cause of Salmonellosis. The dynamics of *Salmonella* infection is variable and are effected mainly by contaminating food and water supplies –especially in

underdeveloped countries-, personal lifestyle, changes in the industry, technology, commerce and travel. [96]. Approximately 2500 Gram-negative *Salmonella* serovars have been reported and can be found in the intestinal tract of both human and animals in nature. *Salmonella* spp. is the first reported biofilm producer foodborne pathogen. This enteric pathogen follows a cyclic lifestyle in which host colonisation is alternated with periods of survival outside the host. The ability of *Salmonella* to form biofilms both host colonisation and non-host conditions contributes to survival and transmission to new hosts [97].

The abiotic surfaces such as plastic, rubber, cement, and stainless steel which are extensively used in farms, food processing industry, kitchens, toilets and bathrooms are the well-documented surfaces on which *Salmonella* is able to form biofilms. Several reports have demonstrated the *Salmonella* biofilms on these abiotic surfaces: According to Stepanovic et al., [98] findings, there was no difference on the ability of biofilm formation on polystyrene microplates of tested 122 *Salmonella* spp. isolates from both humans, animals and food. In accordance with this results, Vestby et al., [99] also found biofilm formation capacity of 111 *Salmonella* isolates feed and fish meal factory environment. It should never be forgotten that toilets and bathrooms in the houses even factories are the most common *Salmonella* contamination source. This fact has been supported by a study [100] which detected survival of *Salmonella* in toilet and bathroom in homes of salmonellosis patients. *Salmonella* spp., *Campylobacter* spp., and *E. coli* O157: H7 are commonly found in meat, poultry and on contact surfaces of meat and ready-to-eat industry due to ample organic residues which could be a niche for microorganism's accumulation and biofilm formation. [17]. The study about biofilm formation by poultry isolates of *Salmonella* on commonly used contact surfaces and their sensitivity to sanitizers was detected [101]. Compatible with this study results Díez-García et al., [102] found the relationships between biofilm formation and growth kinetic parameters of *S. enterica* isolates from poultry. Another study has indicated that there is no significant difference in biofilm-forming ability among the *Salmonella* serotypes (*S.* Typhimurium, *S.* Enteritidis, *S.* Typhi) from different sources or geographical regions [103]. According to a remarkable study's results which detect the effects of different temperature, pH and nutrient availability on *S.* Enteritidis biofilm formation, the environmental stress conditions may alter biofilm resistance to sanitizers [104].

Salmonella is able to adhere to biotic surfaces such as epithelial cells, gallstones and even form biofilms which lead to the development of chronic infections in hosts. Although plants are not considered as a host for enteric pathogens, numerous *Salmonella* outbreaks reported in less developed countries due to contamination of water, soil with sewage. Remarkably, several reports have demonstrated that *S. enterica* can colonise on plants such as sprouted seeds, fresh vegetables and fruits, cilantro; are not killed by surface sterilisation methods [97].

The intrinsic and extrinsic mechanisms involve the biofilm establishment. The extracellular components of *Salmonella* such as flagella, fimbriae (*Pef*), curli fimbria (*Csg*), long polar fimbriae (*Lpf*) and pili are essential equipment for initial attachment, colonisation or cell invasion. The EPS fraction is primarily made by cellulose (encoded by *bcsABZC-bscEFG*), O-antigenic capsule (O-Ag-capsule) and Lipopolysaccharide (LPS) are the sequent fragments. *CsgD* is a major control and integration unit for *Salmonella* biofilm formation which regulates the expression of specific matrix compounds; *RpoS* and *Crl* are the other two main regulators [97].

Another major contaminant in the poultry industry is *Campylobacter* spp. which has similar characteristics with the presence of diarrhoea, cramps [105]. Since *Campylobacter* is present in the industry environment, it can easily form a biofilm on foods, processing areas such as walls, floors, pipes and drains. After the irreversible fixation, *Campylobacter* forms microcolonies which take approximately 4 hours and cells become sessile. The genes *flaA*, *flaB, flaC*, *flhA*, *fliS* encode different flagellar proteins and *cheA*, *cheY* and *CetB* are the responsible for chemotaxis. The QS is activated by the expression of the luxS gene which codes autoinducer-2 synthase. The extrinsic factors such as environmental conditions, the type of available nutrients, and the attachment surface also have effects on the adhesion capacity of *Campylobacter* [106].

E. coli can form biofilms by means of their surface structure as in all members of *Enterobacteriaceae* family. *E. coli* O157: H7 is considered as an enteric pathogen which causes of serious diseases such as haemorrhagic colitis, haemolytic uremic syndrome and thrombotic purpura in humans [107]. The lowest infectious dose (<100 cells) and biofilm forming capacity are the major concerns about *E. coli* O157: H7, this means cross-contamination of foods by food contact surfaces harbouring low numbers of the pathogen can potentially lead to outbreaks [108]. The biofilm formation capacity and degree of *E. coli* O157: H7 on various types of food contact surfaces have been examined in several studies. According to recent survey results, *E. coli* O157: H7 can attach and form a biofilm on wooden surfaces at significantly higher levels compared with steel, glass and plastic and chlorine dioxide show greater lethal activity than NaOCl against *E. coli* O157: H7 in a biofilm on the same surface [108]. Similar findings were demonstrated by Adetunji and Isola [109]. *E. coli* O157: H7 is able to easily adhere and survive on meat contact surfaces even at low temperatures. The presence of other microorganisms on the surfaces is a major factor for the initial attachment. Dourou et al. [110] shown that contact surfaces in the beef fabrication facilities serve as a source of *E. coli* O157: H7 cross-contamination. The type of residual substrate and temperature effect the attachment and attachment occurred not only at a temperature representative of beef fabrication areas during nonproduction hours (15 ^{o}C), but also during cold storage (4 ^{o}C) temperatures.

4. Conclusion

Food preservation is an extensively studied topic in food science. According to these studies results, bacterial elimination can occur either extrinsically or intrinsically. Both extrinsic –influence the food environment to make it non-liveable for microorganisms- and intrinsic- limiting bacterial growth by the direct attack to bacterial- factors contribute to eliminate the microorganisms throughout the food chain. In this respect, the prevalence of microorganisms in biofilm architecture are the major contamination reservoir of both spoilage and pathogenic microflora in the food industry. As discussed above, the ability of pathogenic biofilm formation mainly depends on genetic bases and their regulation, but the properties of the substratum and bacterial cells, environmental conditions such as pH, temperature and nutrient components are the other important factors lead to biofilm formation. As suggested by Meyer [111], there is three significant points to prevent biofilm formation: (1) disinfection before biofilm formation, (2) disinfection biofilm architecture by harsh disinfectants and (3) inhibition of initial attachment by choosing proper surfaces materials which do not allow the bacterial attachment. Because of each biofilm is different due to the diversity of biofilm-forming bacteria in various food industries, the biofilm architecture should be well-analysed to determine an effective cleaning and disinfection operation. The chemical-based control [Sodium hypochlorite (NaClO), Hydrogen peroxide (H_2O_2), Ozone, Peracetic acid], some physical processes such as Hurdle technology, ultrasonication and/or concept of green technology by biological alternatives (natural antimicrobials, phages) are the promising options to inhibiting bacterial quorum sensing and biofilm formation. In this concept, take into account the biofilms must form an essential parameter, while developing the plans such as HACCP, GMP and ISO: 9000 specifications. In order to achieve biofilm-free processing systems, to perform an upgraded HACCP program is necessary for the food industries.

References

[1] WHO Estimates of the Global Burden of Foodborne Diseases 2015; http://apps.who.int/iris/bitstream/10665/199350/1/9789241565165_eng.pdf?ua=1

[2] EFSA (European Food Safety Authority), ECDC (European Centre for Disease Prevention, and Control). Trends and sources of zoonoses, zoonotic agents and food-borne. EFSA J. 2015; 13: 162.

[3] CDC (Center of Disease Control and Prevention). Estimates of Foodborne Illness in the United States. 2014; http://cdc.gov.

[4] Fu, L, Valentino, HR, Wang Y. Bacterial contamination in food production. In: Velazquez JB, editor. Antimicrobial Food Packaging, Elsevier Inc., 125 London Wall, London UK; 2016: 3: 35-43.

[5] Prado, M, Espiña, B, Fernandez-Argüelles, MT, Diéguez, L, Fuciños, P, Vial, S, Oliveira, JM, Reis, RL. 2016. Detection of food pathogens using nanoparticles. Advantages and trends. In: Velazquez JB, editor. Antimicrobial Food Packaging, Elsevier Inc., 125 London Wall, London UK; 14: 183-201.

[6] Havelaar AH, Brul S, Jong AD, Jonge RD, Zwietering MH, Kuile BHT. Future challenges to microbial food safety. International Journal of Microbiology. 2010; 139: 79-94.

[7] Abee T, Kovács AT, Kuipers OP, Veen SVD. Biofilm formation and dispersal in Gram-positive bacteria. Current Opinion in Biotechnology. 2011; 22: 172-179.

[8] Allison DG and Gilbert P. Bacterial biofilms. Science Progress. 1992; 76: 305-321.

[9] Carpentier B and Cerf O. A review: Biofilms and their consequences, with particular reference to hygiene in the food industry. Journal of Applied Bacteriology. 1993; 75: 499-511.

[10] Mattila T, Manninen M, Kylasiurola AL. Effect of cleaning-in-place disinfectants on wild bacterial strains isolated from milk line. Journal of Dairy Research. 1990; 57: 33-39.

[11] Parsek MR and Singh PK. Bacterial biofilms: an emerging link to disease pathogenesis. Annual Review of Microbiology. 2003; 57: 677 - 701.

[12] Russel P. The formation of biofilms. Milk Industry. 1993; 95(9): 10-11.

[13] Sharman M and Anand SK. Biofilms evaluation as an essential component of HACCP for food/dairy processing industry- a case. Food Control. 2002; 13: 469-477.

[14] Costerton JM and Stewart PS. Battling Biofilms. Scientific American. 2001; (285)1: 74-81.

[15] Shi X and Zhu X. Biofilm Formation and Food Safety in Food Industries. Trends in Food Science &Technology. 2009; 20: 407–413.

[16] Sauer K, Rickard AH, Davies DG. Biofilms and biocomplexity. American Society for Microbiology. 2: 2007; 347-353.

[17] Speranza B and Corbo RM. The Impact of Biofilms on Food Spoilage. The Microbiological Quality of Foodborne Spoilers, Woodhead Publishing, ISBN: 978-0-08-100503-3. 2017; 11: 259–282.

[18] Leeuwenhoek AV. Some microscopical observations about animals in the scarf of the teeth. Phil. Trans. 1684; 14: 568-574.

[19] Zobell EC and Eshter CA. The Significance of Marine Bacteria in the Fouling of Submerged Surfaces. Journal of Bacteriology. 1935; 29(2): 239-251.

[20] Zottola EA and Sasahara KC. Microbiol biofilms in the food processing industry- should they be a concern. International Journal of Food Microbiology. 1994; 23(2): 125-148.

[21] Cos P, Tote K, Horemans T, Maes L. Biofilms an extra hurdle for effective antimicrobial therapy. Cur. Parma. Design. 2010; 16: 2279-2295.

[22] Simoes M. Antimicrobial strategies effective against infectious bacterial biofilms. Current Medicinal Chemistry. 2011; 18: 2129-2145.

[23] Akbas MY. Bacterial biofilms and their new control strategies in food industry. In: Méndez-Vilas A, editor. The Battle against Microbial Pathogens: Basic Science, Technological Advances and Educational Programs. Formatex Research Centre; 2013. 114-123.
[24] Sokunrotarak S, Jahid KI, Ha SD. Biofilm Formation in Food Industries: A food safety concern. Food Control. 2013; 31: 572–585.
[25] Bridier A, Sanches-Vizuete P, Guilbaud M, Piard JC, Naïtali M. Biofilm-associated persistence of foodborne pathogens. Food Microbiology. 2015; 45: 167-178.
[26] Stoodly P, Cargo R, Rupp CJ, Wilson S, Klapper I. Biofilm material properties as related to shear-induced deformation and detachment phenomena. Journal of Industrial Microbiology and Biotechnology. 2002a; 29(6): 361-367.
[27] Briandet R, Leriche V, Carpentier B, Bellon-Fortaine MN. Effects of the growth procedure on the surface hydrophobicity of *Listeria monocytogenes* cells and their adhesion to stainless steel. Journal of Food Protection. 1999a; 62: 994-998.
[28] Latasa C, Roux A, Toledo-Arana A, Ghigo JM, Gamazo C, Penades JR, Lasa I. BapA, a large secreted protein required for biofilm formation and host colonisation of *Salmonella enterica* serovar enteritidis. Molecular Microbiology. 2005; 58: 1322-1339.
[29] Van Houdt R and Michiels CW. Biofilm formation and food industry, a focus on the bacterial outer surface. Journal of Applied Microbiology. 2010; 109: 1117-1131.
[30] Donlan RM. Biofilms: microbial life on surfaces. Emerging Infectious Disease. 2002; 8(9): 881-890.
[31] Sinde E and Carbolla J. Attachment of *Salmonella* spp. and *Listeria monocytogenes* to stainless steel, rubber, and polytetrafluorethylene: the influence of free energy and the effect of commercial sanitizers. Food Microbiology. 2000; 17: 439-447.
[32] Costeton JW, Lewandowski Z, Debeer D, Caldwell D, Korber D. Biofilms, the customised microniche. Journal of Bacteriology. 1994; 176(8): 2137-2142.
[33] Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as complex differentiated communities. Annual Review of Microbiology. 2002b; 56: 187-209.
[34] Miller MB, Bassler BL. Quorum sensing in bacteria. Annual Review of Microbiology. 2001; 55: 165-199.
[35] Annous BA, Fratamico PM, Smith JL. Quorum sensing in biofilms: why bacteria behave the way they do. Journal of Food Science. 2009; 74: 24-37.
[36] Yang L, Hu Y, Liu Y, Zhang J, Ulstrup J, Molin S. Distinct roles of extracellular polymeric substances in *Pseudomonas aeruginosa* biofilm development. Environmental Microbiology. 2011; 1705-1717.
[37] Cotter PA, Stibitz S. c-di-GMP-mediated regulation of virulence and biofilm formation. Current Opinion in Microbiology. 2007; 10: 17-23.
[38] Ahmad I, Wigren E, Le Guyon S, Vekkeli S, Blanka A, El Mouali Y, Anwar N, Chuah ML, Lunsdorf H, Frank R, Rhen M, Liang ZX, Lindqvist Y, Romling U. The EAL-like protein STM 1697 regulates virulence phenotypes, motility and biofilm in *Salmonella typhimurium*. Molecular Microbiology. 2013; 90: 1216-1232.
[39] Arvidson S and Tegmark K. Regulation of virulence determinants in *Staphylococcus aureus*. International Journal of Medical Microbiology. 2001; 291: 159-170.
[40] Kon KF, Vuong C, Otto M. *Staphylococcus* quorum sensing in biofilm formation and infection. International Journal of Medical Microbiology. 2006; 296: 133-139.
[41] Linback T, Mols M, Basset C, Granum PE, Kuipers OP, Kovacs AT. CodY, a pleiotropic regulator, influences multicellular behaviour and efficient production of virulence factors in *Bacillus cereus*. Environmental Microbiology. 2012; 14: 2233-2246.
[42] Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. The environmental of cell-to-cell signals in the development of a bacterial biofilm. 1998; Science 280: 295-298.
[43] Boles BR, Thoendel M, Singh PK. Self-generated diversity produces 'insurance effects' in biofilm communities. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101: 16630- 16635.
[44] Kim TJ, Young BM, Young GM. Effect of flagellar mutations on *Yersinia enterolitica* biofilm formation. Applies Environmental Microbiology. 2008; 74: 5466-5474.
[45] Garcia-Gonzalo D and Pagán R. Influence of environmental factors on bacterial biofilm formation in the food industry: a review. Journal of Postdoctoral Research. 2015; 3(6): 3-13.
[46] van der Veen S and Abee T. Mixed species biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum* show enhanced resistance to benzalkonium chloride and peracetic acid. International Journal of Microbiology. 2011; 144: 421-431.
[47] Nilsson RE, Ross T, Bowman JP. Variability in biofilm production by *Listeria monocytogenes* correlated to strain origin and growth conditions. International Journal of Food Microbiology. 2011; 150: 14-24.
[48] Rieu A, Briandet R, Habimana O, Garmyn D, Guzzo J, Piveteau P. *Listeria monocytogenes* EGD-e biofilms: no mushrooms but a network of knitted chains. Applied Environmental Microbiology. 2008; 74: 4491-4497.
[49] Lemon KP, Higgins DE, Kolter R. Flagellar motility is a critical for *Listeria monocytogenes* biofilm formation. Journal of Bacteriology. 2007; 189: 4418-4424.
[50] Houry A, Briandet R, Aymerich S, Gohar M. Involvement of motility and flagella in *Bacillus cereus* biofilm formation. Microbiology. 2010; 156: 1009-1018.
[51] Carpentier B and Cerf O. Persistence of in food industry equipment and premises. International Journal of Food Microbiology. 2011; 145: 1-8.
[52] Botticella G, Russo P, Capozzi V, Amodio ML, Massa S, Spano G, Beneduce L. *Listeria monocytogenes*, biofilm formation and fresh-cut produce. In: Méndez-Vilas A, editor. Microbial pathogens and strategies for combating them: science, technology and education. Formatex Research Center; 2013. 114-123.
[53] Alonso AN, Perrye KJ, Regan PM, Higgins DE. Identification of *Listeria monocytogenes* determinates required for biofilm formation. PLoS One 9. 2014; 12: https://doi.org/10.1371/journal.pone.0113696.
[54] Piercey MJ, Hingston PA, Hansen LT. Genes involved in *Listeria monocytogenes* biofilm formation at a simulated food processing plant temperature of 15 ^{o}C. International Journal of Food Microbiology. 2016; 223: 63-74.

[55] Harmsen M, Lappann M, Knøchel S, Molin S. Role of extracellular DNA during biofilm formation by *Listeria monocytogenes*. Applied Environmental Microbiology. 2010; 76: 2271-2279.
[56] Rodríguez A, Autio W, Mclandsborough LA. Effects of contact time, pressure, percent relative humidity (%RH) and Material type on Listeria biofilm adhesive strength at a cellular level using atomic force microscopy (AFM). Food Biophysics. 2008; 3: 305-311.
[57] Di Bonaventura G, Piccolomini R, Paludi D, D'Orio V, Vergara A, Conter M, Ianieri A. Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food contact surfaces: relationship with motility and cell surface hydrophobicity. Journal of Applied Microbiology. 2008; 104: 1552-1561.
[58] Choi NY, Kim BR, Bae YM, Lee SY. Biofilm formation, attachment and cell hydrophobicity of foodborne pathogens under varied environmental conditions. Journal of Korean Society of Applied Biological Chemistry. 2013; 56(2): 207-220.
[59] Xu H, Zou Y, Lee HY, Ahn J. Effect of NaCl on the biofilm formation by foodborne pathogens. Journal of Food Science. 2010; 75(9): 580-585.
[60] Pilchová T, Hernould M, Prévost H, Demnerová K, Pazlarová J, Tresse O. Influence of food processing environments on structure initiation of static biofilm on *Listeria monocytogenes*. 2014; 35: 366-372.
[61] Tresse O, Lebret V, Benezech T, Faille C. Comparative evaluation of adhesion, surface properties and surface composition of *Listeria monocytogenes* strains after cultivation at constant pH of 5 and 7. Journal of Applied Microbiology. 2006; 101(1): 53-62.
[62] Smoot LM and Pierson MD. Influence of environmental stress on the kinetics and strength of attachment of *Listeria monocytogenes* Scott A and Buna-N rubber and stainless steel. Journal of Protection. 1998; 61(10): 1286-1292.
[63] Tresse O, Lebret V, Garmyn D, Dussurget O. The impact of growth history and flagellation on the adhesion of *Listeria monocytogenes* various strains to polystyrene. Canadian Journal of Microbiology. 2009; 55(2): 189-196.
[64] Herald PJ and Zottola EA. Attachment of *Listeria monocytogenes* to stainless steel surfaces at various temperatures and pH values. Journal of Food Science. 1988; 53(5): 1549-1562.
[65] Moltz AG and Martin SE. Formation of biofilms by *Listeria monocytogenes* under various growth conditions. Journal of Food Protection. 2005; 68(1): 92-97.
[66] Sharma M, Anand SK. Characterization of constitutive microflora of biofilms in dairy processing lines. Food Microbiology. 2002; 19: 627-636.
[67] Salo S, Ehavald H, Raaska L, Vokk R, Wirtanen G. Microbial surveys in Estonian dairies. LWT - Food Science and Technology. 2006; 39(5): 460-471.
[68] Lee SHI, Barancelli GV, de Camargo TM, Corassin CH, Rosim RE, da Cruz AG, Cappato LP, de Oliveira CAF. Biofilm-producing ability of *Listeria monocytogenes* isolates from Brazilian cheese processing plants. Food Research International. 2017; 91: 88-91.
[69] Daneshvar Alavi HE, T Truelstrup HL. Kinetics of biofilm formation and desiccation survival of *Listeria monocytogenes* in single and dual species biofilms with *Pseudomonas fluorescens*, *Serratia proteamaculans* or *Shewanella baltica* on food-grade stainless steel surfaces. Biofouling. 2013; 29: 1253-1268.
[70] López PR, Ibusquiza PS, Mosquera-Fernández M, López-Cabo M. *Listeria monocytogenes* – carrying consortia in food industry. Composition, subtyping and numerical characterisation of mono-species biofilm dynamics on stainless steel. International Journal of Food Microbiology. 2015; 206: 84-95.
[71] Miao J, Liang Y, Chen L, Wang W, Wang J, Li B, Li L, Chen D, Xu Z. Formation and development of *Staphylococcus* biofilm: With focus on food safety. Journal of Food Safety. 2016; 1-11.
[72] Trinetta V, Vaid R, Xu Q, Linton R, Morgan M. Inactivation of *Listeria monocytogenes* on ready-to-eat food processing equipment by chlorine dioxide gas. Food Control. 2012; 26: 357-362.
[73] Park SH, Cheon HL; Park KH, Chung MS, Choi SH, Ryu S, Kang DH. Inactivation of biofilm cells of foodborne pathogen by aerosolized sanitizers. International Journal of Food Microbiology. 2012; 154: 130-134.
[74] Chorianopoulos NG, Tsoukleris TS, Panagau EZ, Falaras P, Nychas GJE. Use of titanium dioxide (TiO_2) photocatalysts as alternative means for *Listeria monocytogenes* biofilms disinfection in food processing. Food Microbiology. 2011; 28: 164-170.
[75] Simões M, Simões LC, Machado I, Pereira MO, Vieira MJ. Control of flow-generated biofilms with surfactants: evidence of resistance and recovery. Bioproducts. 2006; 84(4): 338-345.
[76] Burt S, Essential oils: their antibacterial properties and potential applications in foods. International Journal of Microbiology. 2004; 94: 223-253.
[77] Woo J and Ahn J. Probiotic-mediated competition, exclusion and displacement in biofilm formation by foodborne pathogens. Letters in Applied Microbiology. 2013; 56(4): 307-313.
[78] Minei CC, Gomes BC, Ratti RP, D'Angelis CEM, De Martinis ECP. Influence of peroxyacetic acid and nisin and coculture with *Enterococcus faecium* on *Listeria monocytogenes* biofilm formation. Journal of Food Protect. 2008; 71: 634-638.
[79] Sillankorva S, Oliveira DR, Vieira MJ, Sutherland IW, Azeredo J. Bacteriophage ϕ S1 infection of *Pseudomonas fluoresecens* planktonic cells versus biofilms. BioFouling. 2004; 20: 133-138.
[80] Nostro A, Guerrini A, Marino A, Tacchini M, Di Giulio M, Grandini A, Akin M, Cellini, Bisignano G, Saraçoğlu HT. In vitro activity of plant extracts against biofilm-producing food-related bacteria. International Journal of Food Microbiology. 2016; 238: 33-39.
[81] Folsom JP and Frank JF. Chlorine resistance of *Listeria monocytogenes* biofilms and relationship to subtype, cell density and planktonic cell chlorine resistance. Journal of Food Protect. 2006; 69(6): 1292-1296.
[82] Archer NK; Mazaitis MJ, Costerton JW, Leid JG, Powers ME, Shirtliff ME. *Staphylococcus aureus* biofilms: Properties, regulation and roles in human disease. Virulence. 2011; 2 (5): 445-459.
[83] Arciola CR, Campoccia D, Speziale P, Montanaro L, Costerton JW. Biofilm formation in *Staphylococcus* implant infections. A review of molecular mechanisms and implications for biofilm resistant materials. Biomaterials. 2012; 33: 5967-5982.
[84] Qin Z, Ou Y, Yang L, Zhu Y, Tolker-Nielsen T, Molin S. Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermis*. Microbiology. 2007; 153: 2083-2092.

[85] Lee SHI, Mangolin BLC, Gonçalves JL, Neff DV, Silva MP, Cruz AG, Oliveira CAF. Biofilm-producing ability of *Staphylococcus aureus* isolates from Brazilian dairy farms. Journal of Dairy Science. 2013; 97:1812-1816.
[86] Lopez LAA, Rodrigues JBDS, Magnani M, de Souza EL, de Siqueira-Júnior JP. Inhibitory effects of flavonoids on biofilm formation by *Staphylococcus aureus* that overexpresses efflux protein genes. Microbial Pathogenesis. 2017; 107: 193-197.
[87] Ramírez MD, Smid EJ, Abee T, Groot MNN. Characterisation of biofilms formed by *Lactobacillus plantarum* WCFS1 and food spoilage isolates. International Journal of Food Microbiology. 2015; 207: 23-29.
[88] Simões M, Simões LC, Vieria M A. Review of Current and Emergent Biofilm Control Strategies. LWT - Food Science and Technology. 2010; (43)4: 573-583.
[89] Ladero MDV, del Rio B, Redruello B, Fernández M, Martin MC, Alvarez MA. Biofilm-forming capacity in biogenic amine-producing bacteria isolated from dairy products. Frontiers in Microbiology. 2016; 7: 1-10.
[90] Langsrud S. Biofilm formation by Gram-positive bacteria including *Staphylococcus aureus*, *Mycobacterium avium*, and *Enterococcus* spp. in food processing environments. In Biofilms in the Food and Beverage Industries, Editors Fratamico P, Annous B, Gunther N. Cambridge: Woodhead Publishing, 2009; 250-269.
[91] da Silva Fernandes M, Kabuki DY, Kuaye AY. Biofilms of *Enterococcus faecalis* and *Enterococcus faecium* isolated from the processing of ricotta and the control of these pathogens through cleaning and sanitization procedures. International Journal of Food Microbiology. 2015; 200: 97-103.
[92] Zang QQ, Ye KP, Wang HH, Xiao HM, Xu XL, Zhou GH. Inhibition of biofilms of *Pseudomonas aeruginosa* by an acylated homoserine lactones-containing culture extract. LWT- Food Science and Technology. 2014; 57: 230-235.
[93] Hassett DJ, Korfhagen TR, Irvin RT, Schurr MJ, Sauer K, Lau GW. *Pseudomonas aeruginosa* biofilm infections in cystic fibrosis: Insights into pathogenic processes and treatment strategies. Expert Opinion on Therapeutic Targets. 2010; 14(2): 117-130.
[94] Venturi V. Regulation of quorum sensing in *Pseudomonas*. FEMS Microbiology Reviews. 30(2): 274-291.
[95] Charlebois A, Jacques M, Boulianne M, Archambault M. Tolerance of *Clostridium perfringens* biofilms to disinfectants commonly used in the food industry. Food Microbiology. 2017; 62: 32-38.
[96] Carrasco E, Morales-Rueda A, García-Gimeno RM. Cross-contamination and recontamination by *Salmonella* in foods: A review. Food Research International. 2012; 45: 545-556.
[97] Steenackers H, Hermans K, Vanderleyden J, De Keersmaecker SJD. *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. Food Research International. 2012; 45: 502-531.
[98] Stepanovic S, Cirkovic I, Ranin L, Svabic-Vlahovic M. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Letters in Applied Microbiology. 2004; 38(5): 428-432.
[99] Vestby LK, Moretro T, Langsrud S, Heir E, Nesse LL, Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal and food factories. BMC Veterinary Research. 2009; 5: 20.
[100] Barker J and Bloomfield SF. Survival of *Salmonella* in bathrooms and toilets in domestic homes following salmonellosis. Journal of Applied Microbiology. 2000; 89(1): 137-144.
[101] Joseph B, Otta SK, Karunasagar I, Karunasagar I. Biofilm formation by *Salmonella* spp. on contact surfaces and their sensitivity to sanitizer. International Journal of Food Microbiology. 2001; 64: 367-372.
[102] Díez-García M, Capita R, Alonso-Calleja C. Influence of serotype on the growth kinetics and the ability to form biofilms of *Salmonella* isolates from poultry. Food Microbiology. 2012; 31: 173-180.
[103] Nair A, Rawool DB, Daijad S, Poharkar K, Mohan V, Barbuddhe SB, Kolhe R, Kurkure NV, Kumar A, Malik SVS, Balasaravanan T. Biofilm formation and genetic diversity of *Salmonella* isolates recovered from clinical, food, poultry and environmental sources. Infection, Genetics and Evolution. 2015; 36: 424-433.
[104] Yang Y, Mikš-Krajnik M, Zheng Q, Lee SB, Lee SC, Yuk HG. Biofilm formation of *Salmonella* Enteritidis under food-related environmental stress conditions and its subsequent resistance to chlorine treatment. Food Microbiology. 2016; 54: 98-105.
[105] Rossi DA, Melo RT, Mendonça EP, Monteiro GP, Biofilms of *Salmonella* and *Campylobacter* in the poultry industry. In Poultry Science, Edited Malafi M. InTech, 2017; 94-112.
[106] Moe KK, Mimura J, Ohnishi T, Wake T, Yamazaki W, Nakai M, Misawa N. the mode of *Campylobacter jejuni* biofilm formation on smooth surfaces by. Journal of Veterinary Medical Science. 2010; 72: 411-416.
[107] Doyle MP, *Escherichia coli* O157:H7 and its significance in foods. International Journal of Food Microbiology. 1991; 12: 289-301.
[108] Bang J, Hong A, Kim H, Beuchat LR, Rhee MS, Kim Y, Ryu JH. Inactivation of *Escherichia coli* O157:H7 in biofilm on food-contact surfaces by sequential treatments of aqueous chlorine dioxide and drying. International Journal of Food Microbiology. 2014; 191: 129-134.
[109] Adetunji VO, Isola TO, Crystal violet binding assay for assessment of biofilm formation by *Listeria monocytogenes* and *Listeria* spp. on wood, steel and glass surfaces. 2011; 6: 6-10.
[110] Dourou D, Beauchamp CS, Yoon Y, Geornaras I, Belk KE; Smith G, Nychas GJE, Sofos JN. Attachment and biofilm formation by *Escherichia coli* O157:H7 at different temperatures, on various food-contact surfaces encountered in beef processing. International Journal of Food Microbiology. 2011; 149: 262-268.
[111] Meyer B. Approaches to prevention, removal and killing of biofilms. International Biodeterioration & Biodegradation. 2003; 51(4): 249-253.

Acceptable food products processed from underutilized crops

F.O. Habwe[1], **M.K. Walingo**[2] and **I.J. Jondiko**[3]

[1] Department of Nutrition and Health, Maseno University, P.O. Box 333, 40105-Maseno, Kenya
[2] Maasai Mara University, Kenya
[3] Chemistry Department, Maseno University (Deceased)

Underutilized crops are rich in micronutrients but are only cultivated at subsistence level (1). Food processing technologies, particularly at household level, are challenging and often not applicable to traditional crops (2). Perishability and poor processing are among major constraints facing thier production in developing countries (3). Improved dietary diversity, diet modification and appropriate food processing technologies of micronutrient rich crops are among the long term strategies that could help eradicate or minimize micronutrient malnutrition (4). Processing of underutilized crops into acceptable food products can improve their exploitation. Snack foods like noodles, cookies and crackers are widely adapted for every day use, widely consumed throughout the world and their global consumption is second only to bread (5). This is because they are convenient, easy to cook, low cost and have relatively olng shelf-life (5). Therefore exploitation of the feed value potential of underutilised crops to process crackers, noodles and cookies could increase acceptability and reduce perishability thus increase marketability and consumption/utilisation in the long run. An informal experimental study design was used to evaluate preparation of acceptable food products comprising of crackers, noodles and cookies from cassava (*Manihot esculenta*), finger millet (*Eleusine coracana*), simsim (*Sesamum orientale L.*), and slenderleaf (*Crotalaria ochroleuca* and *C. brevidens*). Product acceptability was assessed using organoleptic tests evaluated by a panel of 76 judges. Disproportionate stratified random sampling was used to select 19 judges from each strata; age strata (<18yrs & >18yrs) and sex strata (men & women) totalling to 76 judges/panellists of 38 females and 38 males. Tests by sensory panels were conducted under controlled conditions using appropriate experimental designs, test methods and statistical analyses according to the International Standards Organization (ISO) (6,78,9). *Chi*-square test was used to analyze the acceptability of the crackers, noodles and cookies across the study groups. The products were highly accepted as over 50% of judges/panellists liked all the food products. This study brings forth acceptable food products prepared from selected underutilized crops. Promotion could lead to increased consumption and marketability of these products. The crackers, noodles and cookies should be promoted and marketed in order to increase consumption and utilization of underutilized crops.

Keywords: Underutilized crops; food products; food processing; food acceptability

1. Food Processing

Reasons for processing food include preservation for use in times of shortage, increase shelf life, removal of toxins, removal of antinutrients which improve digestibility and availability of minerals, and improvement of palatability; food fortification is also part of food processing (10 & 11). Food preparatory practices have changed over time, in East Africa, boiling and occasional roasting were the principal cooking techniques from 1930s to 1960s. These were superior to current practices favouring frying and deep-frying of foods (12). Besides the shift towards frying instead of boiling vegetables and other crops in urban areas, there seem to be no distinctive urban recipes (13). People tend to stick to food habits; thus, preparation techniques acquired in early childhood have a long-lasting influence even if people move from villages to towns (14). Contrary to the above assertions, methods of food processing have been developed over the centuries and are adopted to make the final product more attractive in flavour, appearance, taste and consistency. Besides these aspects of consumer preferences, several of the methods aim at making the food safe and wholesome and increase its shelf life. The common household practices of processing these foods include milling, germinating or sprouting, malting, fermentation and cooking. Each of these processes qualitatively modifies the nutritive value of the food (15). The shelf-life of minimally processed products is increased by pre-processing factors (crop varieties, pre-harvest factors, harvesting, maturity), processing factors (pre-cooling, trimming, washing, cleaning, disinfection, cutting, peeling, handling, dipping, drying, packaging) and distribution conditions (16). Techniques for processing combinations of selected neglected and underutilized crop species into food products have not been established.

An experimental study was carried out at Maseno University Nutrition Department foods laboratory at 65% humidity and 23^{o}C. Here food products (cookies, crackers and noodles) were prepared from underutilized crops which comprised of cassava roots, simsim seeds, fingermillet grains and slenderleaf vegetables. The food products were analyzed for food product acceptability (appearance, smell, taste, texture, and general acceptability) using Organoleptic tests. The underutilized food crops were procured at Luanda market located at 34^{o}36' East and 0^{o} North at an altitude of about 1530 meters above sea level. Same amount (500g) of each crop was procured from three different sellers at different locations of the market and mixed. The procured samples were cleaned and made free from dirt, foreign matter and other stubbles; this was followed by washing using distilled water after which processing was carried out for product preparation. Processing of raw material involved the following activities; fresh vegetable leaves were separated from

roots, washed under running double glass-distilled water. Vegetables were then blanched, drained completely and dried under shade**.**

1.1 Data Analysis

Qualitative and quantitative data analysis was performed using ANOVA with critical significance levels set at $P \leq 0.05$. Data on product acceptability was analyzed using Chi-square test by comparing proportions across the groups (stratum), and Kruskal-Wallis test for comparing differences in continuous data. Post-hoc Dunn's corrections for multiple comparisons were performed following Kruskal-Wallis tests. Acceptable attributes referred to the score values of 4 and 5 on the grading chart while 1, 2 and 3 were for unaccepted attributes.

2. Crop Processing for Product Formulation

2.1 Cassava (*Manihot esculenta*)

Dry cassava was procured and cleaned by picking dirt by hand. This was further washed under running double glass-distilled water to remove dirt then further dried in the open air under the sun. Dried cassava was milled into powder and sieved by a 0.5mm size sieve, then left in the open to release the toxic cyanide which is known to be volatile, in readiness for product processing.

2.2 Fingermillet (*Eleusine coracana*)

Fingermillet was cleaned by removing dirt by hand, this was further washed under running double glass-distilled water to remove dirt then further dried in the open air under the sun. Dried fingermillet was further processed by grilling in the oven to initiate cooking and to produce a characteristic scent. The dried and partially cooked millet was then milled into flour and sieved by a 0.5mm size sieve ready for product processing.

2.3 Simsim (*Sesamum orientale* L.)

Simsim was cleaned by removing dirt by hand further washed under running double glass-distilled water to remove dirt then further dried in the open air under the sun. Dried simism was processed by grilling till golden brown to improve colour and scent. This was followed by grinding then sieving by a 0.5mm size sieve in readiness for product processing.

2.4 Slenderleaf sp. (*Crotalaria ochroleuca* and *Crotalaria brevidens*)

Slenderleaf was destalked washed under running double glass-distilled water to remove surface soil contamination. Vegetables were rinsed three times to remove all the dirt. Each vegetable was processed separately (*ochroleuca* and *brevidens*). After washing, life processes were stopped by immediately blanching. The blanched vegetables were then dried were ground into powder using mortar and pestle and sieved by a 0.5mm size sieve.

The following procedure was followed in blanching the vegetables

1. A large pan half-full of double glass-distilled water was brought to rapid boiling.
2. Clean raw vegetables were put in a wire basket and gently lowered in the boiling water.
3. Once the water began to boil again, vegetables were left in for two minutes.
4. Vegetable basket was removed from the boiling water and plunged into ice-cold water for two minutes to stop the cooking process (over-blanching results into loss of nutrients, flavor and texture).
5. The blanched vegetables were drained and dried under shade until completely dry.

3. Food Products Processing

Formulated recipes followed standard procedures to process the food products according to inventions by (17). However, the wheat flour was not used in these products as cassava was used as a base ingredient and binding agent. This was also meant to break the monotony of using wheat in food product processing. Cassava was combined with slenderleaf to modify cookies, with fingermillet to modify noodles, and with simsim to modify crackers.

3.1 Cookies

Although wheat flour has traditionally been used to prepare cookies, there has been increased need to use other indigenous crops in cookie preparation. African breadfruit was in the past used in combination with wheat flour to prepare cookies (8). Composite sorghum, (yellow Kaura variety)-maize (white flint variety)-wheat flour cookies have been formulated and found to be acceptable in Nigeria (18). On the other hand (8) invented the use of cassava alone to process cassava cookies. This study omitted wheat flour in cookie processing and did not use cassava alone to prepare cookies, instead slenderleaf and cassava flour were combined and used to process cookies. This brings forth a new idea

of using a combination of cassava roots flour and slenderleaf vegetables to prepare cookies (cassava-slenderleaf cookies) besides wheat which has traditionally been used to prepare cookies.

3.1.1 The prepared Cassava-Slenderleaf Cookies

Ingredients

50g	Cassava flour
15g	*C. ochroleuca* powder
12g	*C. brevidens* powder
22g	Margarine
12g	Sugar
1 ¼ tbsp	Salt
50mls	Water

Net weight per serving = **6g**
Total net weight = **100g**

Preparation: The oven was heated to 175^{o}C, the flour, sugar and other dry ingredients were mixed thoroughly then margarine rubbed in to incorporate air. Mixing was continued as water was added till soft then pressed evenly onto bottom of a greased square pan. This was baked for 10 minutes at 175^{o}C. Cookies were then removed from oven and left to cool (19 & 17).
Characteristics: Greenish in colour, dry but easy to break, soft

3.2 Noodles

Attempts have been made to develop nutrient rich noodles by the addition of optimized proportions of wheat and fingermillet flour with other ingredients (20). In addition to these traditional foods, fingermillet is also processed to prepare popped, malted and fermented products like papads, noodles, soups, etc (21). Noodles can also be made from, rice, buckwheat, and starches derived from potato, sweet potato, and pulses, with corn starch as binding agent (22 & 23). Additionally (17), invented the use of cassava alone to prepare noodles. However, in this project noodles were prepared from a combination of fingermillet with cassava thus omitting wheat which is the traditionally known ingredient in processing of noodles. This could break the monotony of using wheat flour in noodle processing and also help promote the use of other underutilized crops like fingermillet in food product processing. This study has revealed the possibility to process noodles from a combination of fingermillet with cassava. Noodles may be used in pasta dish soups, stews, pasta and sauce dishes (19 & 17).

3.2.1 The prepared Cassava-Fingermillet Noodles

Ingredients

50g	Cassava flour
60g	Fingermillet flour
1tsp	Salt
2	Eggs
10mls	Water

Net weight per serving = **10g**
Total net weight = **100g**

Preparation: All dry ingredients were mixed in a bowl and a well made in the centre. Water was added little by little while mixing thoroughly after each addition. Water was added enough to form dough into a very light paste. It underwent kneading for about 10 minutes till smooth and elastic. The dough was then rolled into ¼ inch thin rectangle and cut cross wise into ⅛ inch strips for narrow noodles and ¼ inch strips for wide noodles. The strips were shaken out and placed on a clean dry towel to dry for about 2hrs. When cooking, immerse noodles in boiling water for 10 minutes or until they change from light brown to dark brown as the starch granules gelatinize.
Characteristics: Dark brown in colour, smooth to touch, dry but easy to break, soft after cooking

3.3 Crackers

An invention by (17), indicated the possibility of using cassava alone to prepare crackers. However, banana pulp, pumpkin pulp, mango pulp and mango peel flour have been used to process crackers (24). Results of this study prove that a combination of cassava and simsim can also be used to process crackers, thus including cassava and simsim among crops that can produce crackers.

3.3.1 The prepared Cassava-Simsim Crackers

Ingredients

50g	Cassava flour
50g	Simsim flour
10g	Sugar
10g	Margarine
14mls	Water
1g	Baking powder

Net weight per serving = **8g**
Total net weight = **120g**

Preparation: The oven was preheated to 175^{o}C while a large baking sheet was greased. The flours were then whisked together the flours. Margarine was then beaten together with sugar until light. The flour was then stirred in the flour mixture. Water was slowly poured in and stirred until dough was formed. On a well floured board, the dough was rolled to between ⅛ and ¼ inch thick. A knife was used to cut the dough into desired shapes, baked in the preheated oven for 10 minutes, then removed from oven.
Characteristics: Golden brown in colour, dry but easy to break, soft and crunchy

4. Sensory Analysis of the Modified Food Products through Organoleptc Tests

Tests using sensory panels were conducted under controlled conditions, using appropriate experimental designs, test methods and statistical analyses. A properly designed experimental area and appropriate experimental design, test method and statistical analysis were incorporated in the study (7). Methods mobilized by (6; 8; & 9) were used. The three modified products (cookies, crackers and noodles) were evaluated by a panel of 76 judges. Disproportional stratified random sampling was used to select 19 judges from each strata; age strata (<18yrs & >18yrs) and sex strata (men & women) totaling to 76 judges/panelists (38 females and 38 males). Before each test session panelists were given orientation about the procedure of sensory evaluation, their health status was also considered during selection to avoid those who are pregnant, suffering from colds, and with allergies that affect their sensitivity for the products. Each taster received a maximum of three foods in the session, rated each food as he/she finished it, and rinsed his/her mouth with clean water between samples in order to remove all traces of the previous sample. The sample food products were presented in identical containers with different codes, at the same time. The judges/panelists ranked the products on the basis of sensory visual appearance, taste, smell, texture and overall acceptability using a five point hedonic scale where numbers from 1 to 5 were assigned to the scale's five categories High numbers reflected preference, 5 was assigned the "like extremely"; 4 "like"; 3 "neither like nor dislike"; 2 "dislike"; and 1 "dislike extremely". Score distributions were also used to calculate means and percentages. The three food products comprising of cookies, noodles and crackers were subjected to sensory analysis to assess acceptability using organoleptic test. Products were regarded as acceptable if they had score values of 4 and 5 on the grading chart; on the other hand, 1, 2 and 3 scores were for unaccepted attributes therefore unacceptable products

4.1 Appearance Acceptability of the Modified Food Products

The appearance of crackers was most preferred by all the judges with insignificant differences in the liking of their appearance among judges, they were highly and equally liked by girls, women and boys, and least liked by men. Cookies followed closely in the liking of their appearance with insignificant differences among all the judges. However, they were highly liked by women and least liked by boys. Noodles appearance was the least liked among all the products with insignificant differences among the judges. However, the appearance of noodles was highly liked by girls and least liked by men as indicated in Table.1. Appearance is a very important parameter in judging properly baked biscuits that not only reflect the suitable raw material used for the preparation, but also provides information about the quality of the product (25). The high preference of the appearance of crackers and cookies by judges could therefore be

attributed to the baking processes of the two products as opposed to noodles which were not baked and their appearance was the least preferred among all the three modified food products. The appearance of crackers was most preferred than cookies even though both products were baked. This could be attributed to simsim which is bright in colour used in the preparation of crackers as opposed to slenderleaf which is dark green in colour, used in preparation of cookies. The least preference of noodles' appearance could be attributed to the use of fingermillet which is dark brown in colour. The brighter the colour of product, the higher the appearance acceptability and vice versa.

Table 1 Modified Food Product Acceptability among Study Participants.

Product Attribute	CATEGORY				*df* (χ^2)	P-*value*
	Girls (n=19)	Women (n=19)	Boys (n=19)	Men (n=19)		
Cookie Acceptability, n (%)						
Appearance	14 (73.7)	15 (78.9)	13 (68.4)	14 (73.7)	3 (0.543)	0.909
Taste	16 (84.2)	12 (63.2)	16 (84.2)	14 (73.7)	3 (3.203)	0.361
Smell	12 (63.2)	12 (63.2)	17 (89.5)	15 (78.9)	3 (4.886)	0.180
Texture	15 (78.9)	16 (84.2)	15 (78.9)	17 (89.5)	3 (1.021)	0.796
Cracker Acceptability, n (%)						
Appearance	18 (94.7)	18 (94.7)	18 (94.7)	16 (84.2)	3 (2.171)	0.538
Taste	19 (100)	18 (94.7)	19 (100)	18 (94.7)	3 (2.054)	0.561
Smell	19 (100)	15 (78.9)	15 (78.9)	18 (94.7)	3 (6.428)	0.093
Texture	16 (84.2)	15 (78.9)	14 (73.7)	17 (89.5)	3 (1.751)	0.626
Noodle Acceptability, n (%)						
Appearance	15 (78.9)	14 (73.7)	12 (63.2)	8 (42.1)	3 (6.606)	0.086
Taste	6 (31.6)	4 (21.1)	8 (42.1)	10 (52.6)	3 (4.524)	0.210
Smell	10 (52.6)	7 (36.8)	6 (31.6)	14 (73.7)	3 (8.164)	**0.043***
Texture	16 (84.2)	18 (94.7)	17 (89.5)	17 (89.5)	3 (1.118)	0.773
General Acceptability, n (%)						
Cookies	16 (84.2)	14 (73.7)	17 (89.5)	17 (89.5)	3 (2.375)	0.498
Crackers	19 (100)	19 100)	19 (100)	18 (94.7)	3 (3.040)	0.385
Noodles	15 (78.9)	11 (57.9)	15 (78.9)	18 (94.7)	3 (7.501)	0.058

†% Acceptability – calculated from a score of either 4 or 5 on a five- point hedonic scale, *- Significant

4.2 Taste Acceptability of the Prepared Food Products

Like appearance, the taste of crackers was the most liked by all the judges; they were most liked by girls and boys and least liked by women and men. However, there was insignificant difference in liking of the taste of crackers among the categories. This was followed closely by cookies whose liking for taste was higher among boys and girls and least liked by women while men were the second least. There was insignificant difference in the liking of the taste of cookies among the judges. The taste of noodles was least liked by all the judges, however, among the categories, men were the highest in liking, followed by boys, then girls while women least liked the taste of noodles. However there was no significant difference in their liking among the categories as shown in Table.1. Flavour is the main criteria that makes the product to be liked or disliked (25). Just like appearance, the taste of crackers was the most preferred by all the judges followed closely by cookies while noodles had their taste least preferred by all the judges. High preference of taste in crackers could be attributed to simsim whose taste could have been the contributing factor to its acceptability. This implies that the taste of simsim is most liked compared to slenderleaf and fingermillet used in preparation of cookies and noodles respectively.

4.3 Smell Acceptability of the Prepared Food Products

The smell of crackers was the most liked among all the judges, however, girls were the highest, followed closely by men, then women and boys who least liked the smell at the same percentage. There was no significant difference in the liking of smell of crackers among all judges. Acceptability of smell in cookies was second highest among all the products, however, boys recorded a higher percentage of liking followed closely by men, women and girls on the other hand least liked the smell of cookies but at the same percentage rate. There was no significant difference in the liking of the smell of crackers among all categories. The smell of noodles was the least liked among all the products with significant differences their smell acceptability among the judges. Men highly and significantly liked the smell of noodles, followed by girls, then by women, boys least liked the smell of noodles as indicated in Table.1. The sense of smell is considered to be more defined because an individual requires a relatively high concentration of tastant in order to perceive a taste solution (26). Smell acceptability was higher in crackers compared to cookies and least in noodles. This could be attributed to simsim used in processing crackers. If simsim was the reason for better taste in crackers as

shown in the flavour and taste results, it could have directly contributed to increased smell acceptability of crackers especially due to the fact that they were subjected to baking. Therefore high acceptability of flavor and taste results to increased liking of a product's smell.

4.4 Texture Acceptability of the Prepared Food Products

Despite noodles being the least liked in all the tested attributes, their texture was the most liked among all the products and among all the judges with no significant differences among categories. The liking of the noodles texture was higher among women, followed by men and boys who liked the texture equally while the girls least liked the texture of noodles. The texture of crackers was liked highest among men, followed by girls, then women and finally boys who least liked them. However, there was no significant difference in the liking of the texture of crackers among all the categories. Cookies on the other hand recorded highest texture liking among men, followed by boys and women who liked them equally, while girls least liked the texture of cookies. There was no significant difference in texture acceptability among categories as shown in Table.1. Upon food ingestion the sensors in the mouth detect food texture and consistency, which include mechanical properties (hardness, cohesiveness, adhesiveness, denseness & chewiness); geometrical properties (smooth, gritty, grainy, chalky & lumpy); and moisture properties (juicy, oily or greasy) (27). Although noodles were least preferred in all other attributes, their texture was most preferred among all products and all study participants. This could be attributed to processing procedures of extrusion and drying in the open used during the preparation of noodles as opposed to rolling and baking used in processing cookies and crackers.

4.5 General Acceptability of the Prepared Food Products

Cookies were liked by all judges and there were no significant differences in the liking for cookies among men, women, girls, and boys. However, men highly liked cookies compared to women. Over 68% of all the judges liked all the parameters for cookies (appearance, taste, smell and texture).

Crackers were highly liked by all the girls, women, boys and 94% of men. There was no significant difference in acceptability among the categories. Over 70% of all the judges liked all the parameters for crackers (appearance, taste, smell and texture).

Noodles were highly liked among men and least liked among women. However, there were no significant differences in acceptability among the categories. Generally all the three food products were highly liked by all participants.

Cookies were liked by most study participants thus indicated high acceptability. Generally the proportion of individuals who reported to like cookies was higher among male participants compared to female participants. On the other hand, crackers were highly liked all the girls, women and boys but a few men were undecided about their acceptability. Generally this indicated high acceptability for crackers. Noodles were highly liked by men participants compared to women. However, there was high acceptability of noodles. Generally all the modified food products were liked by all the study participants indicating high acceptability for the products as shown by the results in Table 1.

5. Summary

Although wheat has traditionally been used to prepare cookies, crackers and noodles, this study has demonstrated the possibility of using underutilized crops like fingermillet grains, simsim seeds and slenderleaf vegetables each combined with cassava roots to process noodles, crackers and cookies respectively. The use of these combinations of crops to prepare these food products have not been in existence and have not been documented before.

Baking increased preference for the appearance of cookies and crackers compared to noodles which were dried in the open and recorded least acceptability of appearance. Although both cookies and crackers were baked, the appearance of crackers was the most preferred compared to cookies; this could be due to simsim which is bright in colour used in the preparing crackers compared to slenderleaf which is dark green in colour used in the preparation of cookies. Noodles were the least preferred in terms of appearance and this could also be attributed to the use of fingermillet which is dark brown in colour in its preparation. High preference of taste in crackers could be attributed to simsim used in its formulation which could have contributed to a better tastes compared to slenderleaf and fingermillet used in the modification of cookies and noodles respectively. Smell preference was higher in crackers compared to cookies and least in noodles. This could be attributed to simsim used in cracker preparation. If simsim was the reason for better taste and flavor in crackers as indicated by the flavor/taste results, it could have directly contributed to better smell after crackers were baked. Therefore simsim contributes to the increased acceptability of food products in terms of taste, flavor and smell. Texture preference was higher for noodles among all the judges. This could be to processing procedures of extrusion and drying in the open used in the preparation of noodles as opposed to rolling and baking used in the preparation of cookies and crackers. Therefore extrusion and open drying raises texture acceptability of a food product.

6. Conclusions

1. Neglected and underutilized food crops like cassava, fingermillet, simsim and slenderleaf can be useful in processing food products like noodles, cookies and crackers.
2. Baking improves appearance acceptability of food products.
3. The brighter the color of ingredients used in preparing a food product, the higher the acceptability of product's appearance and vice versa.
4. Use of simsim in food product processing increases taste, appearance and smell acceptability of the end product compared to the use of slenderleaf and fingermillet
5. Extrusion and drying in the open increases preference of a product's texture as opposed to rolling and baking.
6. Among all the modified food products, crackers were highly acceptable followed by cookies and finally noodles. However, all products recorded high acceptability.

7. Recommendations

1. The use of selected underutilized food crops in preparation of food products should be promoted, marketed and recommended for use in food processing industries.
2. The modified cookies, crackers and noodles should be promoted and marketed in order to increase consumption and diet diversity.

Acknowledgements The support by The National Council for Science and Technology, Ministry of Higher Education, Kenya is gratefully acknowledged.

References

[1] Oniang'o R. K., Shiundu, M. K., Maundu, P., & John, S. T. (2005). African Leafy Vegetables. Chennai, India.
[2] Gudrun, G. K., Katja, S., & Irmgard, J. (2013). Production and Processing of Foods as Core Aspects of Nutrition-sensitive Agriculture and Sustainable Diets. *Food Security, 5(6)*, 825-846.
[3] Abukutsa-Onyango. (2007). The Diversity of Cultivated African Leafy Vegetables in Western Kenya. Juja, Kenya: Jomo Kenyatta University of Agriculture and Technology, JKUAT.
[4] Pasricha, S., Drakesmith, H., Black, J., Hipgrave, D., & Biggs, B. (2013). Control of Iron Deficiency Anaemia in Low- and Middle-income Countries. *Blood, 121(14),* 2607-2617.
[5] Onyema, T. C., Ekpunobi, E. U., Edowube, A. A., Odinma, S., & Sokwaibe. E. C. (2014). Quality Assessment of Common Instant Noodles Sold in Nigeria Markets. *American Journal of Analytical Chemistry., 5,* 1174-1177.
[6] Adebe, Y., Stoecker, J. B., Hinds, J. M., & Gates, E. G. (2006). Nutritive Value and Sensory Acceptability of Corn-and Kocho-based Foods Supplemented with Legumes for Infant Feeding in Southern Ethiopia. *African Journal of Food, Agriculture, Nutrition and Development, 6*(1), 1-9.
[7] ISO.8586. (2012). Sensory analysis – General Guidelines for the Selection, Training and Monitoring of Selected Assessors and Expert Sensory Assessors,: ISO.
[8] Ojinnaka, M. C., Anyanwu, F. A., & Ihemeje, A. (2013). Nutritional Evaluation of Cookies Produced from African Breadfruit (*Treculia africana*) Starch and Wheat Flour. *International Journal of Agricultural and Food Science, 3*(3), 95-99.
[9] Singh-Ackbarali, D., & Maharaj, R. (2014). Sensory Evaluation as a Tool in Determining Acceptability of Innovative Products Developed by Undergraduate Students in Food Science and Technology at The University of Trinidad and Tobago. *Journal of Curriculum and Teaching, 3*(1), 10-27.
[10] Hotz, C., & Gibson, R. S. (2007). Traditional Food-Processing and Preparation Practices to Enhance the Bioavailability of Micronutreitns in Plant-based Diets. *Journal of Nutrition, 137*(4), 1097–1100.
[11] Michaelsen, K. F., Camilla, H., Nanna, R., Pernille, K., Maria, S., Lotte, L., et al. (2008). Choice of Foods and Ingredients for Moderately Malnourished Children 6 Months to 5 Years Old.
[12] Raschke, V., Oltersdorf, U., Elmadfa, I., Wahlqvist, M. L., Cheema, B. S., & Kouris- Blazos, A. (2007). Content of a Novel Online Collection of Traditional East African Food Habits (1930s–1960s). *Asia Pacific Journal of Clinical Nutrition, 16*, 140–151.
[13] Abukutsa-Onyango, M., Tushaboomwe, K., Onyango, J. C., & Macha, S. E. (2005, 23rd – 26th November). *Improved Community Landuse for Sustainable Production and Utilization of African Indigenous Vegetables in the Lake Victoria Region.* Paper presented at the Proceedings of the Fifth Workshop On Sustainable Horticultural Production in the Tropics, Njoro, Egerton University.
[14] Sodjinou, R., Agueh, V., Fayomi, B., & Delisle, H. (2009). Dietary Patterns of Urban Adults in Benin: Relationship with Overall Diet Quality and Socio-Demographic Characteristics. *European Journal of Clinical Nutrition, 63*, 222–228.
[15] Singh, P., & Raghuvanshi, S. R. (2012). Finger Millet for Food and Nutritional Security. *African Journal of Food Science, 6*(4), 77-84.
[16] Shewfelt, R. L. (1987). Quality of Minimally Processed Fruits and Vegetables. *Journal of Food Quality, 10*, 143-156.
[17] Slimak, M. K. (1990). United States Patent No. 4,923,709.
[18] Arogba, S. S. (1999). The Performance of Processed Mango (*Mangifera indica*) Kernel Flour in a Model Food System. *Bioresource Technology, 70*, 277-281.

[19] Crocker, B. (1969). *Betty Crocker's COOKBOOK*. New York: Golden Press.
[20] Kulkarni, S. S., Desai, A. D., Ranveer, R. C., & Sahoo, A. K. (2012). Development of Nutrient Rich Noodles by Supplementation with Malted Ragi Flour. *International Food Research Journal 19*(1), 309-313.
[21] Shobana, S., Krishnaswamy, K., Sudha, V., Malleshi, G. N., Anjana, M. R., Palaniappan, L., et al. (2013). Finger Millet (Ragi, *Eleusine coracana* L.): A Review of Its Nutritional Properties, Processing, and Plausible Health Benefits. In *Advances in Food and Nutrition Research* (Vol. 69): Elsevier Inc.
[22] Tan, H. Z., Li, Z. G., & Tan, B. (2009). Starch Noodles: History, Classification, Materials, Processing, Structure, Nutrition, Quality Evaluating and Improving. *Food Research International, 42*, 551-576.
[23] Yalchin, S., & Basmani, A. (2008). Quality Characteristics of Corn Noodles Containing Gelatinized Starch, Transglutaminase and Gum. *Journal of Food Quality, 31*, 465-479.
[24] Aziah, A. A. N. and C. A. Komathi (2009). "Acceptability Attributes of Crackers made from Different Types of Composite Flour." *International Food Research Journal* **16**: 479-482.
[25] Abu-Salem, F. M., & Abou-Arab, A. A. (2011). Effect of Supplement of Bambaras Groundnut (*Vigna Subterraneam L.*) Flour on the Quality of Biscuits. *African Journal of Food Science, 5*(7), 376-383.
[26] Young, J. M., & Trask, B. J. (2002). The Sense of Smell: Genomics of Vertebrate Odorant Receptors. *Human Molecular Genetics, 11*, 1153-1160.
[27] Wilkinson, C., Dijksterhuis, G. B., & Minekus, M. (2000). From Food Structure to Texture: Trends. *Journal of Food Science and Technology, 11*, 442-450.

Acids in grapes

F. Giannetti, **S. Puccioni**, **A.M. Epifani** and **A. Zombardo**

CREA Research Centre for Viticulture and Enology, CREA-VE-LAB Viale Santa Margherita,80 52100 Arezzo, Italy

Beyond water and sugars, acids are important components of grapes with essential implications for taste, wine- making process and preservation. Many acids were found in vine organs but, usually, almost the total content in grape juice is represented by tartaric, malic and citric acid. Their concentration depends on variety, maturation trends and pedo-climatic conditions. To assess the influence of these factors on the acidic content, for three years grape samples from different black and white grapes, cultivated in four different areas were analyzed to determine pH, titratable acidity and the acidic profile by HPLC. The organic acids content was influenced by the cultivar, the production areas and the vintage; however some varieties resulted less susceptible to different conditions.

Keywords: grape juice; organic acids; titratable acidity; HPLC

1. Introduction

Organic acids are characterized by the presence of a carboxyl group that determines their acidic function [1]. Carboxylic acids in plants can also be present as esters and salts and, generally, the vacuolar juice has a weak acidic reaction. Aliphatic organic acids are present in all plants and are responsible of important biological functions such as respiration processes. These acids may reach high concentrations in vegetal tissues. In this case they are related to other regulating system such as the acid-base balance, controlling the formation of cations and anions and creating a "buffer system" that controls, within certain limits, the pH of the medium. In fact, each organic acid, in presence of its salt, constitutes an elastic system designed to avoid any sudden change in the hydrogenation concentration [2].

Grapes acids affect the organoleptic characteristics of juices and wines and are useful in human nutrition stimulating saliva production and contributing to oral hygiene by reducing the number of bacteria responsible for dental caries and oral infections. They also promote the secretion of gastric juices and are slightly laxative and diuretic. When absorbed from the intestine, they pass into the bloodstream and, being weak acids, they have an alkalizing effect. In fact, they easily decompose into carbonic acid, forming sodium and potassium bicarbonates.

The complex of these new molecules is called "alkaline reserve," and constitutes the resource that the body uses to neutralize acids of different origin that are formed during the course of many pathologies. They are also employed for the production of phytocosmetics for skin purifying treatments [3].

When grapes reach the technological maturation, more than 95% of total acids is represented by tartaric, malic and citric acid [4, 5]. Grape must also contains mineral acids such as sulfuric, hydrochloric and phosphoric, which rarely exceed the concentration of 1 g/L. These strong acids are completely neutralized and are present in the form of ionized neutral salts such as calcium magnesium potassium sulfates, chlorides and phosphates, etc.

The concentration of the acidic functions of free and semi-salified acids forms the acidity of the musts, and it can be determined by titration with an alkaline solution up to pH 7. The titratable acidity is usually expressed as g/L of tartaric or sulfuric acid or milliequivalents per liter [6]. The salified fraction of organic acids requires a more complex procedure to be determined: the analysis of the alkalinity of ashes. The perceived acidity, when we taste a wine or a must, depends directly on the concentration of H^+ ions and then on the pH value. Tartaric acid is always present in small quantities in plants, except for a few species such as tamarind and grapevine, where it is the most abundant acid. In particular, grape tartaric acid is in the dextrorotatory optically active form with levo configuration (L(+) tartaric acid) [8] having the formula: $HOOC(CHOH)_2COOH$ and M.W. = 150,09.

It is the strongest acid in grapes and at technological ripeness its concentration ranges between 2 and 8 g/L, depending on the cultivar, on agronomic management of the vineyards and on pedo-climatic factors.

It is a bi-acid and, in aqueous solution, has two dissociation constant: pK1=3,04 and pK2=4,37. It has two secondary alcohol groups which give it particular physico-chemical properties: the acid strength and the solubility in water of the acid and its salts. In particular, potassium bitartrate is not soluble in presence of ethanol and can cause undesired precipitates in wines. Malic acid, contrary to tartaric acid, is very common in the *Plantae* kingdom and it is present in all kind of fruits. It is particularly abundant in apples, plums, pears, peaches and apricots. Natural malic acid is the L (-) malic isomer, having the formula $HOOCCH_2CHOHCOOH$ and M.W. = 134,09. The malic acid content in ripe grapes can vary according to the grapevine variety, to seasonal trends and to temperatures during the last stages of maturation. Its average concentration ranges between 1,5 and 4 g/L. It is a bi-acid and has two dissociation constants in water, which are respectively: pK1 = 3,46 and pK2 = 5,13. The acid strength is lower than that of tartaric acid. It is always soluble in musts and wines but it can undergo microbiological transformations. Citric acid is a tri-acid having the formula HOC (COOH) $((CH_2)\ COOH)_2$ and M.W. = 192,13. It is present in many fruits and in particular in the citrus *genus*, in healthy grapes its concentration ranges between 150 and 500 mg/L, but it can reach higher values in case

of *Botrytis cinerea* infections. Its dissociation constants are in order: 3,13, 4,76 and 6,40. In musts and wines conditions, with pH values between 3,20 and 3,90, it behaves like a bi-acid with an intermediate strength between tartaric and malic acid. It is always soluble in musts and wines but can undergo microbiological transformations. Due to its structure, it is able to form chelates with iron and copper ions, giving to musts and wines higher stability preventing the formation of hazes and precipitates known as iron and copper "casses".

Other acids that can be found in grapes only in little amounts or traces are: shikimic, ascorbic, aconitic, α-ketoglutaric, fumaric, galacturonic, glyceric, glycolic, isocitric, oxalic, oxaloacetic and pyruvic acids.

In this paper the content of malic, tartaric and citric acid in grapes from 8 different cultivar grown in 4 different areas of Tuscany (Italy) were compared to evaluate how different environmental conditions can affect the acids concentrations.

2. Materials and methods

The grapes were collected from four different areas of Tuscany (Italy), two from vineyards near the coast, in the provinces of Pisa (PI) and Grosseto (GR), and two from vineyards in the provinces of Arezzo (AR) and Lucca (LU). For the trial were chosen both local and international varieties, their names and abbreviations are listed in Table 1.

Table 1 List of white and black grape cultivars and their relative abbreviations.

Black grapes	White grapes
Cabernet sauvignon (Cab)	Manzoni bianco (Man)
Sangiovese (San)	Pinot bianco (PiB)
Pinot nero (PiN)	Sauvignon (Sau)
Nero d'Avola (NdA)	Fiano (Fia)

The vineyards had a density of 3600 plants per hectare, with 2,5 m of distance between rows and 1,1 m on the row. The vines, with a permanent unilateral cordon, were spur-pruned upward vertical position. Grapes were harvested and analyzed in 2014, 2015 and 2016 seasons. Titratable acidity and pH were measured following official EU methods (Official Methods of Wine Analysis, Reg. 440/2003). The concentration of malic, tartaric and citric acid were determined by HPLC as described by Flamini et al. [7]. Analysis of variance (ANOVA) was performed using Statgraphics Centurion (Ver.XV, StatPoint Technologies, Warrenton, VA).

3. Results

Table 2 F-Ratio and P-value for chemical parameters analyzed for grape samples.

	Titratable acidity		pH		Tartaric acid		Malic acid		Citric acid	
	F-Ratio	P-Value	F-Ratio	P-Value	F-Ratio	P-Value	F-Ratio	P-Value	F-Ratio	P-Value
Factors										
A: Cultivar	16,4	< 0,001	62,3	< 0,001	25,7	< 0,001	8,5	< 0,001	3,9	0,002
B: Zone	2,3	n.s.	17,5	< 0,001	27,5	< 0,001	29,7	< 0,001	0,9	n.s.
C: Year	25,6	< 0,001	6,5	0,004	46,6	< 0,001	18,7	< 0,001	3,2	0,050
Interactions										
AxB	2,3	0,012	2,4	0,001	4,8	< 0,001	0,6	n.s.	1,3	n.s.
AxC	1,1	n.s.	1,8	n.s.	3,5	< 0,001	3,2	0,002	1,3	n.s.

The data on acidic composition of grapes were processed with the analysis of variance (ANOVA) considering as factors the cultivar, the year of production, the area of origin and their interactions.

The titratable acidity was mostly affected by the factors year of production (F = 25,6) and cultivar (F = 16,4). The values ranged between 5g/L and 7g/L and in 2014, a year characterized by low temperatures and frequent rains, the level of titratable acidity was higher than in 2015 and 2016. Although the production area did not have significant effects on the average values, the effect of its interaction with the cultivar was significant (P=0,001), showing the behaviour of the varieties in different pedo-climatic conditions. In details, the titratable acidity of PiN, PiB and Man grapes was constant in all experimental vineyards, whereas in other varieties, San, Sau and Cab, it was highly influenced by the Zone factor (Tab. 2, Fig. 1).

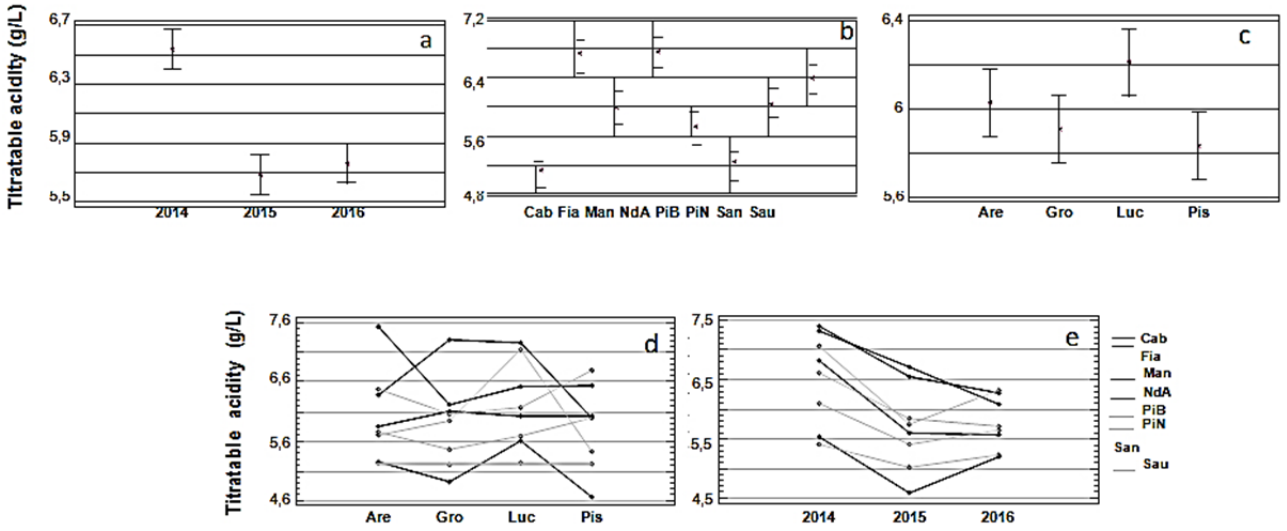


Fig. 1 Average values of titratable acidity, as g/L of tartaric acid, per factors (a: Year, b: Cultivar, c: Zone) and interaction plot of Variety with Zone (d) and Year (e).

The average pH values in the three-year period showed small variations with lower levels in 2015 and in the zone LU. Considering the cultivars, the pH varied approximately from 3,5 in Cab and PiN and 3,0 in Fia. The interaction between cultivar and zone resulted significant and confirmed the trends observed for titratable acidity (Fig. 2).

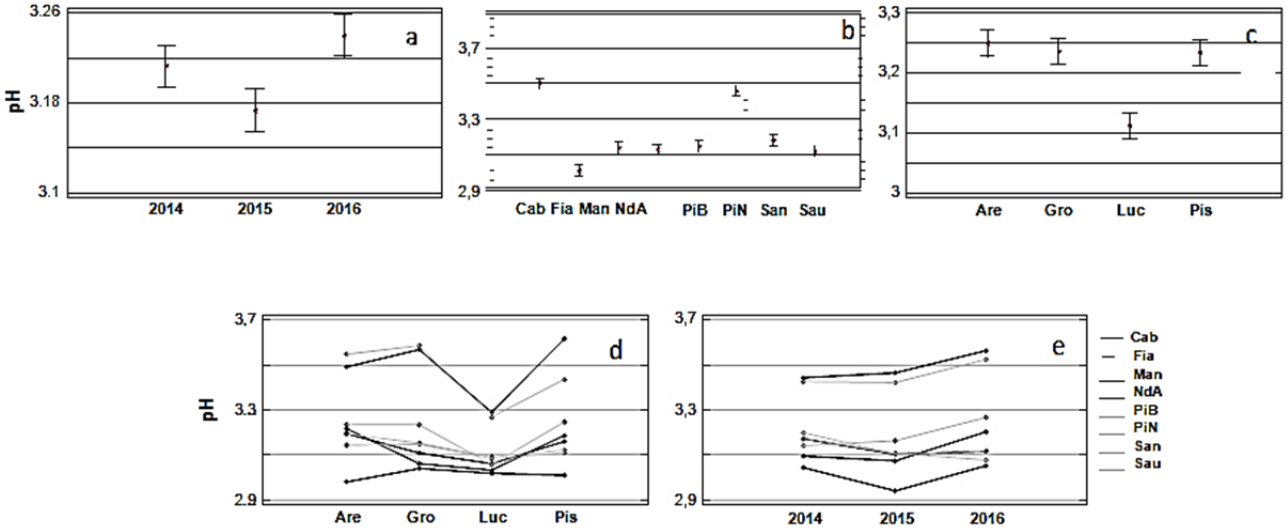


Fig. 2 Average values of titratable pH per factors (a: Year, b: Cultivar, c: Zone) and interaction plot of Variety with Zone (d) and Year (e).

The concentration of tartaric acid was strongly influenced by all the factors, and the interactions were also significant (Tab. 2). The average values ranged from about 3 g/L in the Arezzo and Pisa area and 3,7 g/L of Lucca (Fig. 3). Regarding the cultivar, the values were between 2,5 g/L of Cab, PiN and PiB and 4 g/L of NdA. During the three years of observation, mean values dropped from 3,7 g/L in 2014 to 2,9 g/L in 2016. Each variety had constant concentrations in all the vineyards except for Sangiovese and Nero d'Avola grown in the Lucca hills, that produced grapes with higher tartaric acid content than in other zone.

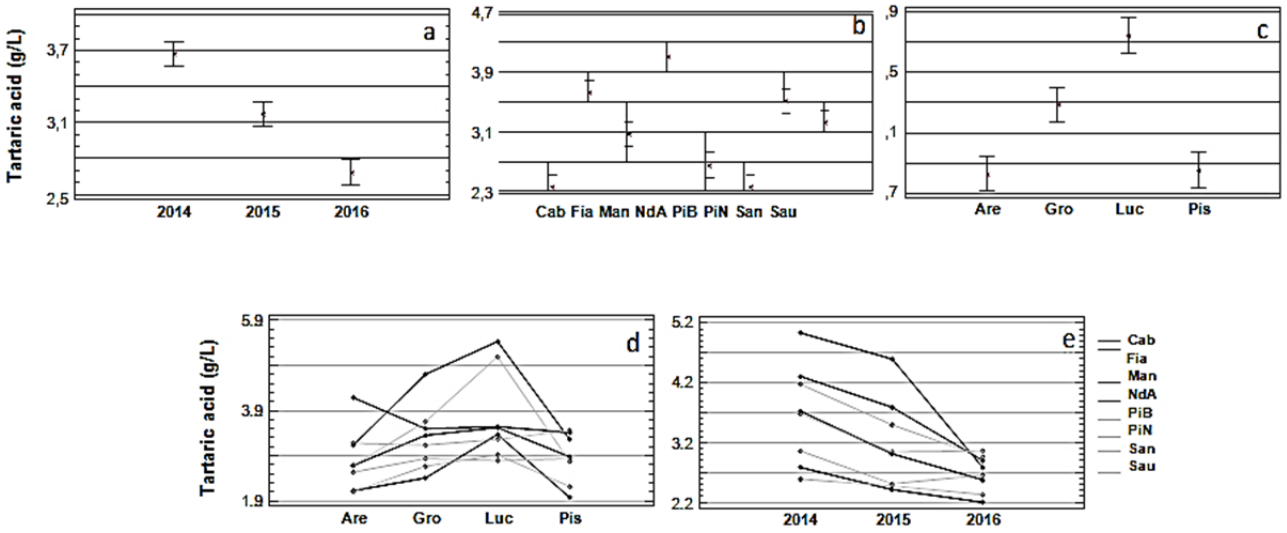


Fig. 3 Average values of tartaric acid per factors (a: Year, b: Cultivar, c: Zone) and interaction plot of Variety with Zone (d) and Year (e).

Malic acid showed average values below 3 g/L and it was always lower than tartaric acid. As it was observed for tartaric acid, in 2014 malic acid concentrations were higher than in the 2015 and 2016 vintages that resulted, in this case, similar to each other. The vineyards of Grosseto and Lucca, despite the grapes showed higher tartaric acid values than the others, resulted the areas where the lowest malic acid concentrations were measured. White berry grapes were richer in malic acid; the values ranged between 2,3 g/L of Sauvignon and 1,7 g/L of Sangiovese and Nero d'Avola. In 2015, differently than the other varieties, Nero d'Avola and Cabernet sauvignon incurred in a decrease of the malic acid average concentration. Finally, all the variations were influenced in the same way in the different cultivation areas, the interactions between Variety and Zone, in fact, were not significant (Tab. 2, Fig. 4).

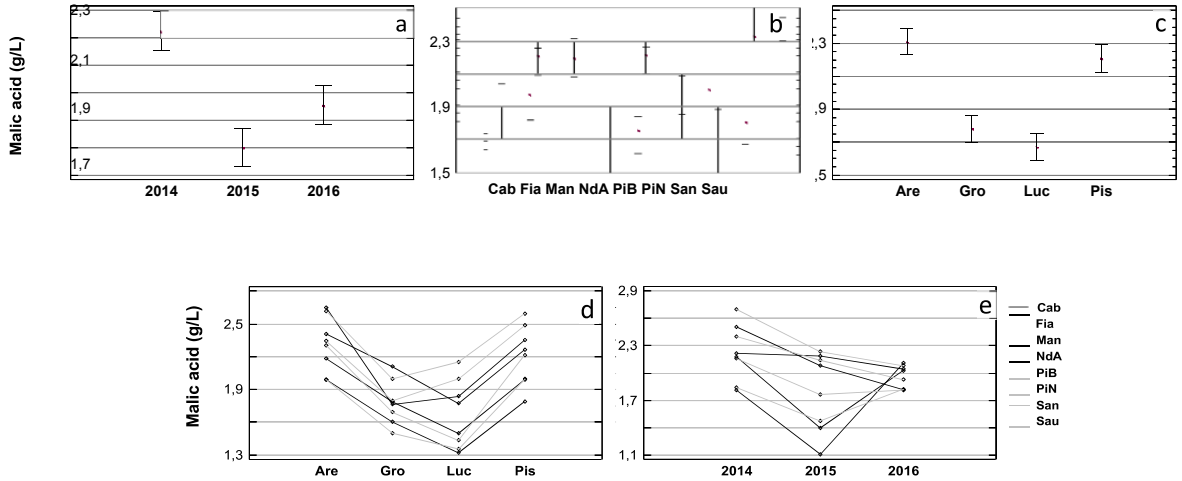


Fig. 4 Average values of malic acid per factors (a: Year, b: Cultivar, c: Zone) and interaction plot of Variety with Zone (d) and Year (e).

Citric acid, as expected, was the minor acid in all the examined grapes, and it was always lower than 0,5 g/L. The data varied within 0,41 g/L of Manzoni bianco and 0,31 g/L of Nero d'Avola and Fiano. The factors Year and Zone had little effect on citric acid concentrations; the interactions between the factors do not reveal significant variations and the differences were modest (Tab. 2, Fig. 5).

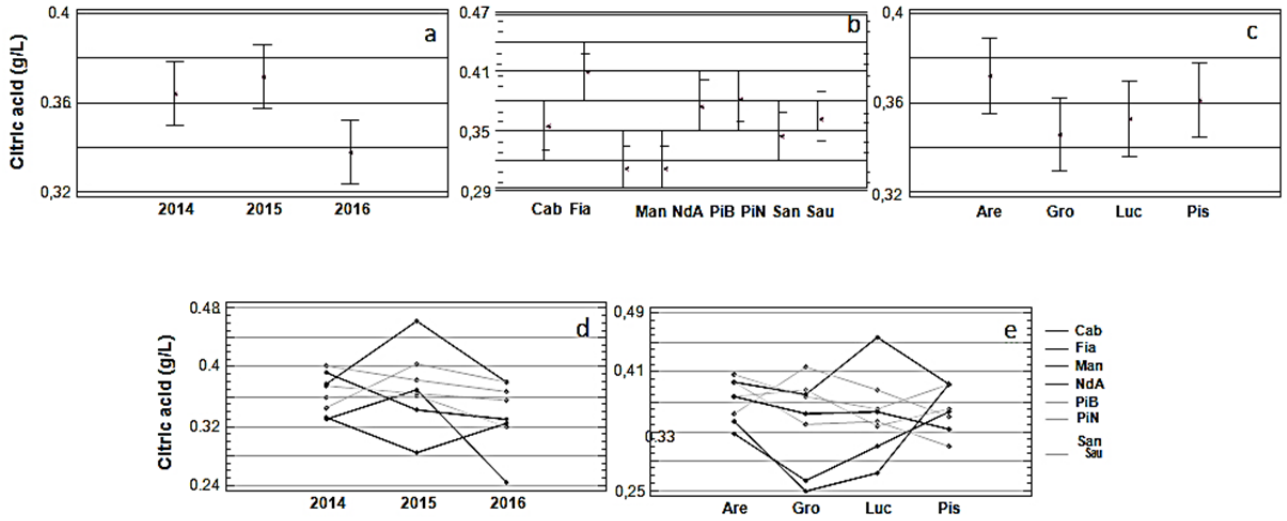


Fig. 5 Average values of citric acid per factors (a: Year, b: Cultivar, c: Zone) and interaction plot of Variety with Zone (d) and Year (e).

4. Conclusions

The three-year analysis showed that titratable acidity was higher in 2014, a year characterized, in Tuscany, by a fresh and rainy summer that caused a delayed maturation of fruits and provoked, consequently, an increase of the concentration of the major acids.

The grapevine varieties considered showed differences in acidic composition. Cabernet Sauvignon, Pinot n. and Pinot b. had the lowest levels of tartaric acid, while higher contents were detected in Nero d'Avola and Sangiovese, which at the same time showed lower levels of malic acid.

From the results emerged that some grape varieties have a more steady behaviour and are less dependent on the factors Year and Zone (PiB, PiN and Man), while others are much more unstable (NdA and San). Tartaric acid content was influenced by all the factors considered, but a more pronounced effect was caused by the Year. Malic acid is also more affected by environmental factors (Zone and Year). The concentrations of citric acid were only marginally conditioned by pedo-climatic conditions, while it seems to be typical of the cultivar.

Since organic acids play a fundamental role in the taste and nutritional properties of grapes and wines, it is important to know which mechanisms regulate the accumulation in the berries.

This work has highlighted how the variability in organic acid content in grapes can be strictly related to the response of the different varieties to the environmental factors. Only with comparative studies on many cultivars it can be determined which are the specific factors that actually affect the quality of the grapes. This preliminary work represents only a small example and, in perspective, it will be repeated increasing the number of variables and grape varieties involved, and expanding the analytical spectrum of the monitored metabolites, such as the acids that usually are present in grapes in smaller quantities or in trace amounts.

References

[1] Ternay A.L., Contemporary organic chemistry. W.B. Saunders company. Philadelphia; 1979. p. 769-778.
[2] Cultrera R., Chimica agraria. Ed. Cedam Padova 1977; Vol I, p. 129-156.
[3] Various Authors, Enciclopedia della salute. Ed. Librex, Milano; 1971 . Vol. VI, p. 1642-1644.
[4] Cotea V.D., Tratat de oenologie. Ed. Ceres, Bucarest 1985; Vol II, p. 197-215.
[5] Wen Y., Cui J., Zhang Y., Duan C., Pan Q. 2014. Comparison of organic acids levels and L-IdnDH expression in Chinese-type and European-type grapes. Euphytica 196: 63-76.
[6] Blouin J., Guimbertau G., Maturation et Maturité des raisins. Ed. Feret, Bordeaux; 2000. p.55-65.
[7] Flamini R., Hyphenated techniques in grape and wine chemistry. J.Wiley & Sons Ltd Chicester England; 2008. p. 1-11
[8] Usseglio-Tomasset L. Chimica enologica. Edizioni AEB Brescia 1978; p. 31-37.

Acrylamide in Processed Food and Reduction Strategies

G. Arusoglu

Department of Nutrition and Dietetics, School of Health Sciences, Kirklareli University, Kayali Kampüsü. Kirklareli, Turkey

Objective: After determining that acrylamide (AA) occurred in carbohydrate-rich foods through frying and roasting as a result of heat treatment, protein-rich foods have also been found to occur in lower levels of acrylamide today. Acrylamide formation in studies of high-risk foods are French-fries and chips, biscuits, bread and cereals. Its formation is also identified in protein content rich food in Turkish cuisine such as döner, kebap, meat and poultry products. The International Agency for Research on Cancer has classified acrylamide in food as a 'Group 2 Possible Carcinogenic to Humans'. Due to the AA evaluated as potential carcinogens, numerous studies have been done to reduce AA formation in cooked products. This article aims to provide a better understanding of the chemistry and biology of acrylamide in food, and can lead to the development of food processes to decrease the acrylamide content in the diet.

Methods: Medline, Embase, Cochrane Central Register of Controlled Trials databases were used for searching acrylamide information.

Conclusions: Various heat treatments applied in food for preparation, processing and storage such as frying, baking, sterilization, roasting, grilling and even drying can lead to physical and chemical changes. Studies of AA have reported that it is important to determine the formation of AA in carbohydrate and protein-rich foods after heat application over 120ºC. It occurs at lower concentrations in protein rich foods (eg. döner, kebap, grilled meat and chicken) and is not formated in raw and boiled food.

Comment: The total amount of acrylamide in food can be reduced significantly by suitably processing as well as raw materials added to the product.

Keywords: Acrylamide in food; heat treatment; asparagine; maillard reaction

1. Introduction

Acrylamide may have a risk for public health. Acrylamide (AA, 2-propenamid was first found by Christian Moureau in Germany as a chemical compound in 1893 [1]. Acrylamide is the most active compound which has been researched among food contaminants, on which acrylamide heat effect has been applied [2]. Thereafter, many researchers have released publications stating that they found Acrylamide at different amounts in several food substances [1, 3]. The existence of chemical acrylamide in food was published in a press release in April 2002 by the Swedish National Food Administration (SNFA) and Stockholm University. According to that release, it is presented to the attention of the public that *"carbohydrate rich foods"* contain high degrees of acrylamide which is a chemical substance that has the potential to cause cancer in fried and baked foods [4, 5]. The International Cancer Research Center (IARC, 1994) classifies acrylamide in the "2A group", that is, "it is a potential carcinogen for people".

Product variables and process variables are two factors that influence the formation of AA. While the product variables are things such as asparagine, sugar type and concentration, pH, water content and the food matrix, the process variables are things such as the temperature and the time.

Acrylamide influences the genotoxic, carcinogenic, neurotoxic, reproduction and development toxicity [6]. According to the classification system of the European Union (EU), AA has toxic effects on the carcinogenic, mutagen and reproduction [7, 8, 9].

According to the WHO directive for drinking water quality, it is stated that 1 liter drinking water contains 0.5 µg acrylamide. This figure in the European Union is 0.1µ g/lt water [10]. Acrylamide has been found to be carcinogenic with a standard 2 years bioassay works in lab rats. It is known that it causes benign and malign tumors. AA has been included in the drinking water of lab rats at 2 mg/kg/day in two independent animal work groups and it has been approved that it is carcinogenic. At the same time, this result is valid for brain tumors, spinal cord and other tissues [10].

The objective of this article is to focus on the formation mechanism of the acrylamide found in some products and provide complied information or the strategies to decrease it in food.

Materials and Methods: Medline, Embase, Cochrane Central Register of Controlled Trials databases were used for searching acrylamide information.

2. Formation of Acrylamide in Foods

Acrylamide has two forms which are monomeric and polymeric [11]. Acrylamide is one of the elements that have a negative effect on human health and are formed as a result of heat treatment. Acrylamide is a water-soluble vinyl

monomer used in the synthesis of the polyacrylamides and involves polar functional groups in different physical and chemical characteristics and the monomer form is created in food [3, 12, and 13]. This monomer is an amide, involving unsaturated double bonds and it appears as a crystal while it is white and shows resolution at room temperature. It is odor free and has high solubility in water. It is polymerized immediately once it is dissolved or exposed to oxidative agents. The heat of the solution is 84.56^{o}C and the boiling temperature is 1256^{o}C. It is a unique compound since its polymeric form is a water proof gel [1].

It has been indicated that the monomeric form of acrylamide has a toxic effect on the nervous system and has an anemic effect as well and it is a carcinogen in lab animals [1, 11]. It is possible that the level of acrylamide in the diet can form higher than the other known carcinogens. Formation of acrylamide depends on the temperature and time and the ambient temperature needs to be in excess of 100^{o}C for acrylamide to form. It has been reported that acrylamide has been formed by a reaction of specific amino acid with reducing sugar (Maillard reaction) during the formation of browning around 120 ^{0}C. Furthermore, formation of AA is affected by the period of cooking, food source, and type of the food and cooking temperature. The World Health Organization (WHO) and Food and Agriculture Organization (FAO) state that foods which are processed or cooked at high temperatures can considerably contain AA and this can cause a risk for the health of the people [14]. When the ambient temperature increases to 180^{o}C, the formation of the acrylamide maximizes.

3. Probable Mechanisms

Even if all of the formation mechanisms of acrylamide are not known, there are positive results as to the formation mechanism. Alternatively, it can react to form the "Schiff base", which expresses the Maillard reaction with free sugar and free amino acids. It has been put forward that during the formation mechanism of the acrylamide, the Schiff base is formed, which is an indecisive by-product, from the reaction of asparagines with carbonyl after the decarboxylation in the Strecker type reaction. Acrylamide is formed with 3- aminopropanamid with hydrolysis of the decarboxlate Schiff base and thereafter the dissolution of ammoniac.

Besides, the high degree lipids that are generated due to reducing lipids (blown oil acid or glycerol) pave the way for the formation of AA. Acrolein is a three carbon aldehyde and resembles the structure of acrylamide. Acrolein enters into the oxidation reaction and generates an acrylic radical by-product. AA is generated upon the existence of the two type nitrogen resources but acrolein is not required in the alternative formation mechanism of the AA [15]. For this reason, acrolein is accepted as the beginning of acrylamide. Acrolein can turn into acrylamide with basic chemical transformations [16]. Acrylamide is not constituted in fat, which are used in deep frying in the food production industry [17].

Sugar alcohols or polyols (sorbitol, xylitol) do not react to Maillard reactions. This means that when baked products are sweetened with sorbitol, the color does not or rarely changes during baking. The reaction of large sugar is slower with amino acids. Pentose sugars (5 carbon atom), such as ribose, react faster than the hexose sugars (glucose, fructose) and disaccharide (sugar, lactose). With its two amino groups, lysine from the amino acids, reacts faster and causes the formation of a brown color. The formation of the Maillard reaction is avoided since some flavor compounds are formed in some circumstances such as sterilized dairy products. An excessive heat application may result in the loss of quality since it changes the structure and flavor as well as odor compounds. The loss of essential amino acids such as lysine due to the Maillard reaction significantly affects the biological values of the proteins in foods [18].

It has been observed that the asparagine rate in the asparagus is at a high level (11000-94000 mg/kg) and AA may be formed at high rates in asparagus under the proper circumstances in the resources which state that asparagine is the major pioneer of acrylamide in foods [19, 20].

No acrylamide is formed when glucose, glycine and cystine or methionine amino acids are heated to 185°C [21]. They only form acrylamide at trace amounts when glutamine and aspartic acid are exposed to heat (0.5–1 mg mol-1) [21].

This work has been undertaken by the Marmara Research Center (MAM), affiliated with the Scientific and Technological Research Council of Turkey (TUBITAK). It has been observed that acrylamide is found in the crust, which is largely consumed by the Turkish people and no acrylamide formation is available in the bread [22]. TUBITAK observed that rice, tahini halva, kebab, doner kebab, grills, rye bread have AA at lower than the measurable amount and French fries and several bakery products have the highest rates in Turkey. According to the recent research, meat and poultry products such as döner, grills and kebab, which have an important place in Turkish cuisine, have been examined in terms of the acrylamide content and the level of acrylamide has been observed between the ranges of 25-250µg/kg [23].

4. Factors Affecting the Formation of Acrylamide in Foods

The existence and concentration of molecules such as asparagines and reducing sugars have influence on the formation of AA. The heat, heat density and water activity, which are used in the processing technologies and relative

concentration of these molecules, have effects on the formation of AA. Asparagine concentrations and reducing sugars for each of the three products have been affected by the cultivation conditions (season, watering and fertilization) harvest season and storing conditions. Redundancy of the reducing sugars and asparagine concentration for cereal affects the formation of AA. On the contrary, asparagines are seen more in potatoes and reducing sugar concentration affect the formation of AA [24].

AA formation in green beans of coffee is not affected by reducing the sugar content and shows a weak correlation with the asparagine concentration. During coffee roasting, AA is not accumulated and its formation and degradation (deformation) happen concurrently. While free asparagine seems to be a limiting factor for the formation of the acrylamide, the concentration of the reduced sugar is affected less. 20% of the AA which has been formed in the beginning, is formed during roasting. Roasted and ground coffee contains 170–351 μg/kg AA. The subsequent degradation is observed during storing. It is reported that acrylamide mainly occurs for the affiliation with the coffee ground compounds and roasted coffee matrix. It is required to take more notice of such degradation since the amount changes depending on the period of the storing to guess the exposure rate. For that reason, it is recommended to have a moderate coffee consumption [21]. No relation has been observed between the amount of sugar and AA formation during the baking or roasting process [25].

According to gender, age and ethnic group, the suggestion is to consume between 0.3 and 0.8 μg/kg. According to the 2002 evaluation by WHO and FAO, AA exposure per day is 0.3-0.8 μg/kg body weight [26]. There is a requirement to drastically decrease exposure since there is no threshold value of the effect for genotoxic carcinogens. According to WHO, cancer risk is 1/100.000 when we are exposed to AA 1μg/day throughout our life. Risk assessment of these compounds, containing the toxicological and exposure status has to be conducted [26].

5. Precautions to Decrease the Formation of Acrylamide in Food

Well baked products contribute to the total AA intake due to containing AA concentrations. Decreasing the cooking period or heat in formation of Acrylamide effectively diminishes the formation of AA [27, 28]. The studies about decreasing the formation of acrylamide cover the characteristics of the raw material. For example, the effect of conditions such as the harvest year, variety, storage conditions in potato products on the level of the acrylamide has become a subject for many studies. Furthermore, there are many findings that antioxidants decrease the formation of acrylamide in food which has heat treatment at high temperatures applied [29].

To sum up, refined cereal products increase cereal consumption. Well baked products contribute to the total AA intake due to them containing AA concentrations. Decreasing the cooking period or heat in formation of Acrylamide effectively diminishes the formation of AA. Gertz and Klostermann reported that the formation of AA accelerates over 175^{o}C. It is stated that the formation of AA decreases by 50% when the temperature of the frying oil is decreased from 185^{o}C to 165°C in French fries; and when it is decreased from 190^{o}C to170^{o}C, it decreases by 68% and from 190^{o}C to 150^{o}C, formation of AA decreases by 88% [26, 30].

To sum up, it has been stated that elements such as potato type, the agriculture system, vaccination, damp, heat density, enzyme and the addition of some minerals and amino acids, carbonyl (R-CHO), the concentration of the compounds, harvest time (seasonal changes), asparagine amount, pH, cooking rules, additives, storing period and the degree and vaccination have a significant effect on the formation of AA concentration [26, 31].

6. Discussion

According to the evaluation by the World Health Organization (WHO) and FAO in 2002, exposure to acrylamide is 0.3-0.8 μg/kg/day body weight. Neither value measurement over the predictions nor biological process is understood well. A mechanism has yet to be found to stop the formation of acrylamide. It is known that AA is constituted in some foods which are cooked or processed at high temperatures and it increases with the waiting period at a high temperature.

In brief, acrylamide can be formed through different mechanisms, which contain the reactions of carbohydrates, protein and amino acids, lipids or other micro food elements in food treated with heat. As it is observed in the studies, to control the level of acrylamide is to start from the selection of the raw material of which the product will be manufactured. There can be a significant decrease in the amount of acrylamide with the optimization of the processing conditions and suitable raw material. Cooking conditions to be carried out on food should be systematic and common analysis methods should be developed.

It is recommended for individuals to take adequate and balanced nutrition, to increase their food and vegetable consumption, decrease total fat intake and fried food consumptions. It goes without saying that people who are living in Turkey are very lucky to have a culture of braising in the cooking pot.

References

[1] Boettcher MI, Schettgen T, Kütting B, Pischetsrieder M, Angerer J. Mercapturic acids of acrylamide and glycidamide as biomarkers of the internal exposure to acrylamide in the general population Mutation Research 2005;580:167–176.

[2] Zhang Y, Zhang G, Zhang Y. Occurrence and analytical methods of acrylamide in heat-treated foods. Review and recent developments. Journal of Chromatography A. 2005; 1075:1–21.

[3] EFSA. Food standards agency study of acrylamide in food background information and research findings press briefing. What is acrylamide? 2002.

[4] Dearfield KL, Abernathy CO, Ottley, MS, et al. Acrylamide. Its metabolism, developmental and reproductive effects, genotoxicity and carcinogenicity. Mutation Research/Reviews in Genetic Toxicology. 1988;19581:45-77.

[5] Vattem DA, Shetty K. Acrylamide in food: a model for mechanism of formation and its reduction. Innovative Food Science and Emerging Technologies. 2003;4:331-338.

[6] Abramsson-Zetterberg L, Vikstrom AC, Törnquist M, Hellenas KE. Differences in the frequency of micronucleated erythrocytes in humans in relation to consumption of fried carbohydrate-rich food. Mutation Research 2008;653:50–56.

[7] Friedman M, Mottram D. Chemistry and Safety of Acrylamide in Food. Volume 561, Advances in Experimental Medicine and Biology. 2005; p161-163.

[8] Ruden C. Acrylamide and cancer risk-expert risk assessments and the public debate. Food and Chemical Toxicology. 2004;42:335-349.

[9] Zhi-Rong L, Zou HC, Park SJ, Park D, Shi L, Oh SH, Park YD. The effects of acrylamide on brain creatine kinase: Inhibition kinetics and computational docking simulation. International Journal of Biological Macromolecules. 2009; 44:128–132.

[10] Joint FAO/WHO Consultation on Health Implications of Acrylamide in Food (2002: Geneva, Switzerland). Health implications of acrylamide in food: Report of a joint FAO/WHO consultation, WHO Headquarters, Geneva, Switzerland, 25-27 June 2002.

[11] Lopachin RM, Schwarcz AI, Gaughan CL. In vivo and in vitro effects of acrylamide on synaptosomal neurotransmitter uptake and release. NeuroToxicology. 2004; 25:349-363.

[12] LoPachin RM. The Changing View of Acrylamide Neurotoxicity. NeuroToxicology. 2004; 25:617–630.

[13] Zhang Y, Zhang Y. Formation and Reduction of Acrylamide in Maillard Reaction: A Review Based on the Current State of Knowledge. Critical Reviews in Food Science and Nutrition. 2007; 47:521–542.

[14] Gokmen V, Senyuva H. A simplified approach for the kinetic characterization formation in fructose-asparagine model system. Food Additives and Contaminants. April, 2006; 23(4): 348–354.

[15] Taeymans D, Wood J. A Review of Acrylamide: An Industry Perspective on Research, Analysis, Formation, and Control. Critical Reviews in Food Science and Nutrition. 2004; 44:323–347.

[16] Lingnert H. Acrylamide in food–mechanisms of formation and influencing factors during heating of foods. The Swedish Institute For Food and Biotechnology Chairman of Expert Committee. 2002.

[17] Totani N, Yawata M, Takada M, Moriya M. Acrylamide Content Of Commercial Frying Oil. Journal of Oleo Science. 2007; 56(2) :303-306.

[18] Skog K. The HEATOX Project. Heat-generated food toxicants: identification, characterisation and risk minimisation. Final report. Lund University; 12 April 2007.

[19] Kamal-Eldin A. Lipid Oxidation Pathways (Ed.), AOCS Press, Illinois. 2003.

[20] Mottram DS, Low MY, Elmore JS. The Maillard reaction and its role in the formation of acrylamide and other potentially hazardous compounds in foods, in: K. Skog, J. Alexander (Eds.), Acrylamide and Other Hazardous Compounds in Heat-Treated Foods,Woodhead Publishing Limited, Cambridge. 2006.

[21] Becalski A, Lau BPY, Lewis D, Seaman SW. Acrylamide in foods: Occurrence, Sources and Modelling. Journal of Agriculture and Food Chemistry. 2003; 51(3):802-808.

[22] CIAA. Confederation of the food and drink industries of the EU. The CIAA acrylamide toolbox. Rev 9. 2006.

[23] Kaplan O, Kaya G, Özcan C, İnce M, Yaman M. Acrylamide concentrations in grilled foodstuffs of Turkish kitchen by high performance liquid chromatography-mass spectrometry. Microchemical Journal. 2006; 93:173–179.

[24] Klaunig JE. Acrylamide Carcinogenicity. J. Agric. Food Chem. 2008;56:6162–6166.

[25] Seal C J, De Mul A, Eisenbrand G, Haverkort A, Franke Knut J, Lalljie Sam PD, et. al. Risk-benefit considerations of Mitigation Measures on Acrylamide Content of Foods- A Case study on Potatoes, Cereals and Coffee. British Journal of Nutrition. 2008; Ap. Vol 99: Supp 2.

[26] Dybing E, Saner, T. Risk Assessment of Acrylamide in Foods. Toxicological Sciences. 2003; 75:7– 15.

[27] Pelucchi C, Galeone C, Levi F, Negri E, Franceschi S, Talamini R, et al. Dietary acrylamide and human cancer. Int. J. Cancer. 2006; 118:467–471.

[28] Ou S, Lın Q, Zhang Y, Huang C, Sun X, Fu L. Reduction of acrylamide formation by selected agents in fried potato crips on industrial scale. Innovative Food Science and Emerging Technologies. 2008; 9: 116-121.

[29] Gertz C, Klostermann S. Analysis of acrylamide and mechanisms of its formation in deep-fried products. Eur. J. Lipid Sci. Technol. 2002;104:762–771.

[30] Haase NU, Matthaeus B, Vosmann K. Acrylamide formation in foodstuffs - minimizing trategies for potato crisps. Dtsch. Lebensm. Rundsch. 2003; 99:87–90.

[31] Saldamlı İ. [Food Chemistry] Gıda Kimyası. Hacettepe Üniversitesi Yayınları. 2007.

Application of Near Infrared Spectroscopy (NIRs), PCA and PLS models for the analysis of dried medicinal plants

J. Gajdoš Kljusurić, **D. Valinger**, **A. Jurinjak Tušek**, **M. Benković** and **T. Jurina**

Department of Process Engineering, University of Zagreb, Faculty of Food Technology and Biotechnology, Pierottijeva 6, 10 000 Zagreb, Croatia

In traditional medicine, botanicals and medicinal plants in their natural and processed form are widely used [1] due to their medicinal and antioxidant properties. Numerous analytical methods have been developed for the analysis of chemical composition of medicinal plants extracts like gas chromatography (GC), mass spectrometry (MS), thin layer chromatography (TLC), UV spectrometry, and high performance liquid chromatography (HPLC). All these methods are precise but expensive, time-consuming and require many reagents. As an alternative, near infrared spectroscopy (NIRs), as a simple, selective, and environmentally friendly method [2], can be used.

NIR spectroscopy is a non-destructive measurement method that allows intact measuring, without any additional sample preparation or pre-treatment. Use of spectroscopy in the near infrared region allows a wide range of applications in the food chain production, from control of raw materials to intermediary and final products [3] in order to provide a quality guarantee for consumers.

NIR spectroscopy is based on the electromagnetic absorption in the near infrared region. Spectral analysis has to be assisted with various chemometric techniques, such as multiple linear regression analysis (MLRA), principal component analysis (PCA) and partial least squares regression (PLSR) [4]. Chemometric techniques and chemometric modelling have become an integral part of spectral data analysis which also includes pre-processing of NIR spectra. The pre-processing objective is removal of physical phenomena in the spectra in order to improve the subsequent multivariate regression, classification model or exploratory analysis [5].

In this work, most widely used pre-processing techniques including (i) scatter-correction methods and (ii) spectral derivatives are explained through analysis of spectra of dried medicinal plants collected during the size reduction process (milling), as well as during analysis of the kinetics of the solid-liquid extraction process using water as a solvent [6]. In order to identify patterns in large set of data and express the data to highlight similarities and differences among them, PCA was used. PCA presents the pattern of similarity of the observations and the variables by displaying them as points in maps [7]. PLS regression was used to predict or analyse a set of dependent variables from a set of independent variables or predictors. The predictive ability of a PLS model is expressed as one or more statistical measures. Which parameter should be used is described by R-Squared Coefficient, Ratio of standard error of Performance to standard Deviation (RPD) and Range Error Ratio (RER).

Keywords: near infrared spectroscopy; data pre-processing, modelling, principal component analysis, partial least squares regression

1. Introduction

Our fast-paced modern lifestyle leaves us very little time for regular nutrition and care for our health leading to increased occurence of chronical disseses such as cancer, vascular diseases and neurodegenerative diseases. Medicinal plants have been used since ancient times for treatment of a wide range of diseases and are the richest bioresource of drugs for traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs [8]. Since medicinal plants represent a rich source of biologically active compounds that have a beneficial effect on health, a wide range of medicinal plants can be found on the market today. Studies have been carried out globally to verify their efficacy and some of the findings have led to the production of plant-based medicines. Some of the health benefits of medicinal plants include antimicrobial and antioxidant efficacies, which can be of great significance in therapeutic approaches of many diseases [9]. Up to this day, there have been several studies documenting the antibacterial, antifungal, antiviral, anticancer and anti-inflammatory properties of plant ingredients [10, 11, 12].

With the rapid increase in sale and variety of medicinal plants there is a growing concern for quality of the products present on the market. Although there are many methods for quality control such as HPLC, GC, MS, TLC etc., there is still demand for less expensive, consistent, rapid and non-destructive method. A significant number of literature reports have emerged over the past 20–30 years regarding the use of near infrared spectroscopy (NIRS) as both a research tool for formulation development and as a quality control technology for monitoring unit operations for product manufacturing [13].

It is well known that NIR spectra contains both chemical and physical information (such as particle size and bulk density) [14, 15, 16] thus regular or in-line particle size measurement can be one of the ways to control the quality of certain products where medicinal plants are used as raw materials or as extracts for their production. The aim of this

study was to examine the possibility of differentiating particle size of the same plants and the different particle size of different plants and also to show the potential of Partial Least Squares Regression (PLSR) models for prediction of different parameters such as conductivity, dry matter, total disolved solids (TDS) and total polyphenols (TP) based on NIR spectra. The non-invasive spectroscopy in the near infrared range of 904 - 1699 nm (NIR) was used to record spectra of five different medicinal plants (sage, lavender, lemon balm, thyme, mint) for seven different particle sizes (<100 μm, 100 μm, 250 μm, 355 μm, 500 μm, 800 μm and 1000 μm) that were further used for diferentiation of different particle sizes by Principal Component Analysis (PCA). Except crude medicinal plants, water extracts of medicinal plants were also investigated (marigold, sage, lemon balm, thyme, St. John's wort and lavender) by PCA. PLS regression modelling was performed on lavender water extract as part of study for the project „Application of microreactors in the analysis of antioxidant activity of medicinal plants (MICRO-AA)", project number HR.3.2.01-0069, which was funded by the European Social Fund (ESF) through the Human Resources Development program.

Results have shown that NIR spectroscopy was successful in differentiating particle sizes of different plants and highly sensitive to different particle sizes of the same plants. PLS regression modelling results show a very promising potential in monitoring certain physical and chemical properties of medicinal plants water extracts by NIR spectroscopy.

2. Materials and methods

2.1 Materials

2.1.1 Plant material

Dried plant materials of sage (*Salvia officinalis* L.), lavender (*Lavandula angustifolia* L.), lemon balm (*Melissa Officinalis* L.), thyme (*Thymus vulgaris* L.), mint (Mentha piperita L.), marigold (Calendula officinalis) and St. John's wort (Hypericum perforatum) collected in the north-western part of Croatia during the flowering season of 2015, dried and properly stored, were purchased from a specialized herbal store (Suban d.o.o., Strmec, Croatia).

2.1.2 Chemicals and reagents

Folin-Ciocalteu's reagent and sodium carbonate were purchased from Kemika (Zagreb, Croatia). Trolox (6-hydroxy-2,5,7,8-tetra methylchromane-2-carboxylic acid) and potassium peroxodisulphate were obtained from Fluka (Buchs, Switzer land). ABTS•+ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)diammonium salt), gallic acid (3,4,5-trihydroxybenzoic acid) were obtained from Aldrich (Sigma–Aldrich Chemie, Steinheim, Germany), and ethanol (96%) was obtained from Carlo Erba Reagents (Cornaredo, Italy).

2.2 Methods

2.2.1 Milling

Dried plant material of sage, lavender, lemon balm, thyme and mint was milled using IKA control Tube mill (IKA-Werke, Staufen, Germany). Milling conditions were adjusted as follows: 20 000 min^{-1} for 40 seconds. Since the plant material had different organic structures and thus different hardness, for some samples milling time was reduced to 20 seconds. After milling, samples were kept in a desiccator until further used.

2.2.2 Separation of particle size fractions

In order to separate the particle size fractions milled plant material was subjected to sieving. Used sieves had 100 μm, 250 μm, 355 μm, 500 μm, 800 μm and 1000 μm pore opening. Seven different particle size fractions were obtained marked as <100 μm, 100 μm, 250 μm, 355 μm, 500 μm, 800 μm and 1000 μm which were then subjected to NIR analysis.

2.2.3 Extraction procedure

An amount of 2 g of dry plant material of marigold, sage, lemon balm, thyme, St. John's wort and lavender was placed in a 150 mL glass with 100 mL of deionised water and heated to 80 °C using Ika HBR4 digital oil-bath (IKA-Werk GmbH & Co.KG, Staufen, Germany). Selected temperature and extraction conditions (t = 90 min, rpm = 500 min^{-1} and T = 40 °C) were chosen for water extracts of six medicinal plants (marigold, sage, lemon balm, thyme, St. John's wort and lavender) on which seven parameters: pH, conductivity, dry matter, TDS, TCP, AO and BR-AO were measured. For the PLSR models of lavender selected temperature and extraction conditions were: t = 90 min, rpm = 500 min^{-1} and T = 60 °C. After the extraction process samples were immediately cooled in the water-ice mixture, then filtered through

a 100 % cellulose paper filter (LLG Labware, Meckenheim, Germany) with 5 – 13 μm pore size and stored at T = 4 °C until analysed.

2.2.4 Light microscopy

Light microscope imaging was performed on the milled plant material samples in order to identify size, structure and type of particles present in the samples. Samples were viewed by a Motic B1 Series microscope (Motic, Kowloon, Hong Kong) at 4x magnification and photographed by a Moticam 3 microscope camera (Motic, Kowloon, Hong Kong).

2.2.5 Determination of pH, conductivity, total dissolved solids (TDS) total phenol content (TPC), dry matter and antioxidant capacity measured by DPPH method (AO) and Briggs-Rauscher method (BR-AO)

All the analyses were performed as described in the work of Jurinjak Tušek et al., 2016 [17] except Briggs-Raucher method for antioxidant capacity which was performed as described in the work of Gajdoš Kljusurić et al., 2005 [18].

2.2.6 NIR spectroscopy

The NIR spectra (range extends from λ = 904 nm to λ = 1699 nm) of medicinal plants for different particle sizes and water extracts were collected with the setup for NIRS studies that included: a laptop, NIR-128-1.7-USB/6.25/50μm scanning monochromator from Control Development, Inc., provided with Spec32 software, the polychromatic source of light, optical cables, and a hemispherical cup that serves as a sample tray. Complete NIR instrument setup has been previously described in detail by Valinger et al. (2011) [19]. For dried plant material, small metal vessel was used while the water extracts were poured in quartz cuvette of 1 mL volume. No mechanical or chemical treatment of the samples was needed prior to NIRS measurements.

2.2.7 Spectral analysis and chemometric models

Principal Components Analysis (PCA) was used for identifying patterns in data and expressing the data to highlight similarities and differences. PCA also represents the pattern of similarity of the observations and the variables by displaying them as points in maps [20, 21, 22]. Data obtained by NIRS were used to perform principal component analysis (PCA) by means of statistical software StatSoft STATISTICA v. 10 (StatSoft Inc., Palo Alto, USA) and were plotted in 3D using Wolfram Mathematica v10 (Wolfram Research, Champaign, USA).

PLS regression was used to predict a set of dependent variables from a set of independent variables or predictors. This prediction is achieved by extracting from the predictors a set of orthogonal factors called latent variables which have the best predictive power. PLS regression is particularly useful when we need to predict a set of dependent variables from a (very) large set of independent variables (i.e., predictors) [23]. PLSR modelling was carried out using Unscrambler® X 10.4, trial version software (CAMO software, Oslo, Norway).

3. Results and discussion

3.1 Near infrared (NIR) spectra

For five different medicinal plants (sage, lavender, lemon balm, thyme, mint), 6 recordings of NIR spectra were made for each particle size (<100 μm, 100 μm, 250 μm, 355 μm, 500 μm, 800 μm i 1000 μm), which resulted altogether in 42 recordings per medicinal plant (210 spectra in total). Example of unprocessed spectra for different particle sizes of lavender are presented in Figure 1.

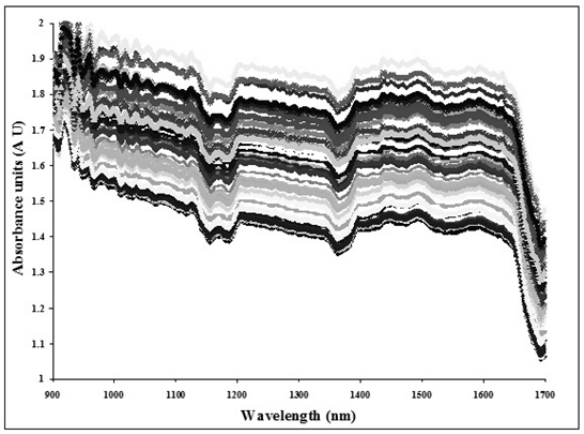


Fig. 1 NIR spectra of different particle size fractions (<100 μm, 100 μm, 250 μm, 355 μm, 500 μm, 800 μm i 1000 μm) of lavender.

3.2 Application of chemometric tools

From the raw spectra, it is impossible to extract information without using at least one of the chemometric methods. Based on the final aim, most common used chemometric tools are Principal Component Analysis (PCA) and the Partial Least Squares (PLS) regression. PCA analysis is a variable reduction technique that is used when the main aim is grouping or detection of similarities/differences between the observe variables, while PLS regression is used when the main aim is bearing of some relation between the response and independent variables. Both of the most commonly used multivariate tools were applied to present their usefulness. As an independent data set, NIR spectra of different medical plants were used.

3.2.1 Effectiveness of use of Principal component analysis

To investigate the effectiveness of distinction of medical plant(s) based on their particle size, and to determine the detection limit, PCA analysis was applied. Using Principal Component Analysis (PCA) for each medicinal plant and all the particle size fractions, cluster analysis was made, and example of results for two medicinal plants (sage and lemon balm) are presented in Figure 2A and 2B. Results are presented in term of first two principal components where the pronounced effect of the first two eigenvalues is observed. For the results presented in Fig 2 A) and 2 B) cumulative effec[illegible] the first two principal components.

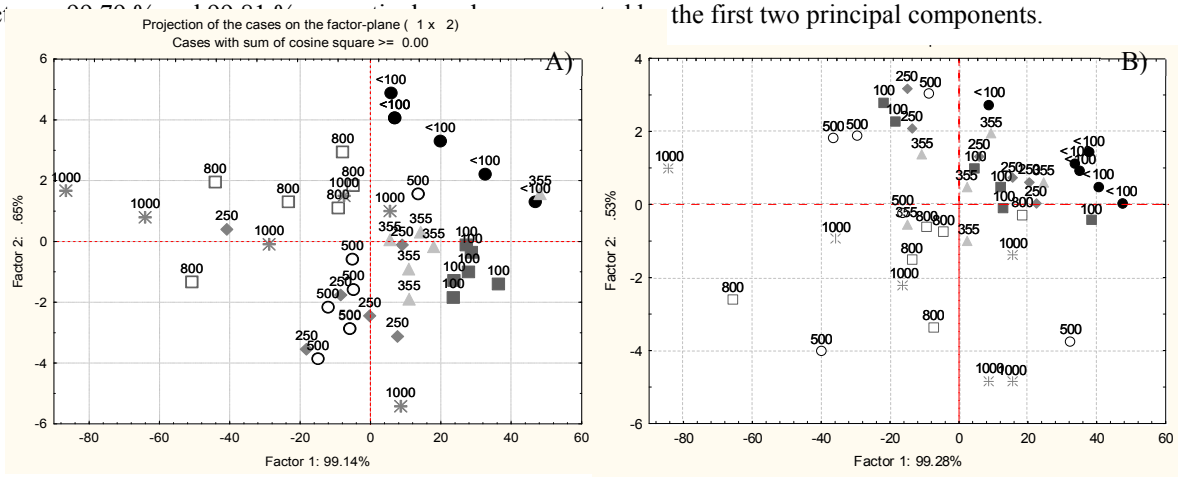


Fig. 2 Principal Component Analysis of NIR spectra presented by first two factors for particle size fractions (<100 μm, 100 μm, 250 μm, 355 μm, 500 μm, 800 μm i 1000 μm) of A) sage and B) lemon balm.

Figures 2A) and 2B) show that NIR distinguished different fractions of the same medicinal plant for some medicinal plants with better precision (lemon balm) while others with lesser precision (sage). Similar results were obtained in the work of Valinger et al. [24], where distribution of different particle size fractions of medicinal plants (chamomile, dandelion, nettle, broadleaf plantain and yarrow) was investigated. As in this case, smaller particle fractions (<100 μm, 100 μm) showed good uniformity and clustering, while overlaping of larger particle size fraction was observed. To explain this overlaping microscopic images of larger particle sizes fractions were made and example of two medicinal plants (lavander and sage) for particle sizes fractions of 800 μm are shown if Fig. 3A) and 3B).

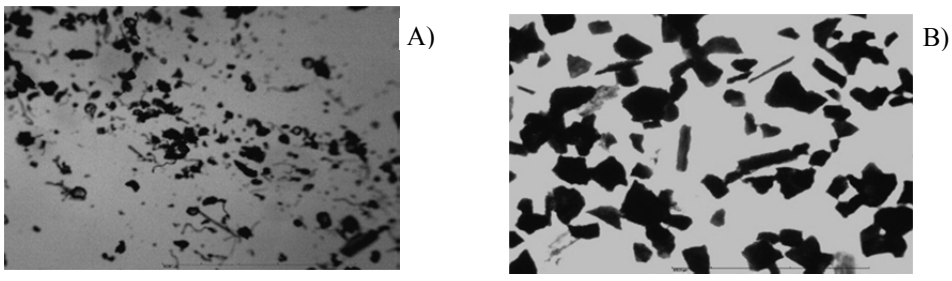


Fig. 3 Images of ground samples for particle sizes of 800 μm for A) lavender and B) sage (magnification 4x)

In Figs. 3A) and 3B) it is visible that samples of sage and lemon balm fractions of 500 μm were not homegenous because there are smaller and larger particles present in desired size fraction. That is the reason why PCA did not cluster every fraction separately. If there is uniformed particle size distribution with the use of NIR spectroscopy it is possible to completely separate different particle size fractions as described in work of Valinger et al. [25]. Because of the NIRS sensitivity, if there are few larger/smaller particle sizes in certain fraction NIRS will remove this sample from the

cluster which shows its potential in the quality control and falsification control of different food products. If, for example, the calibration was done with two different particle size fractions in term of the experiments with different ratios of fractions ranging from 0 % of fraction A and 100 % of fraction B to 100 % of fraction A and 0 % of fraction B it could be possible to determine how much of one fraction was present in the mixture.

To investigate the effectiveness of PCA analysis in distinguishing different medical plants of the same particle size, NIR spectra (NIRS) of 5 different medical plants (sage, lavender, lemon balm, thyme, mint) was analysed. Those plants are often used in Croatia as tea preparations in prevention of different diseases such as gums and throat problems, gastritis and stress. PCA was also used to confirm that NIRS can distinguish different medicinal plants that have the same fraction size. In Figure 4 A) and 4 B) PCA of same particle size fractions (<100 μm and 100 μm) for five different medicinal plants (sage, lavender, lemon balm, thyme, mint) is presented in term of first two principal components (Factor 1 & Factor 2).

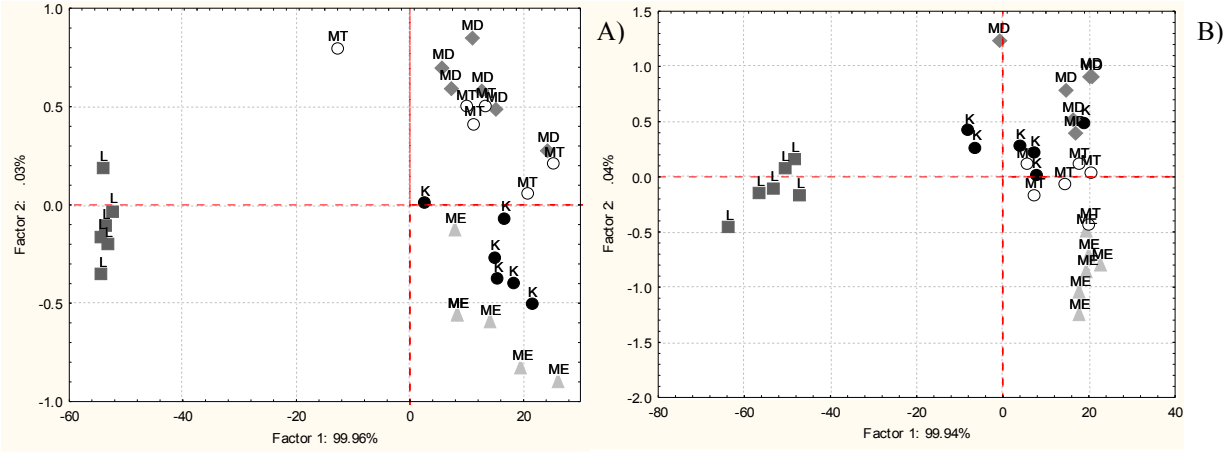


Fig. 4 Images of ground samples for particle sizes of <100 μm A) and 100 μm B) for five different medicinal plants (K - sage, L - lavender, MD – lemon balm, ME – thyme, MT – mint)

Sometimes using PCA for first two factors overlapping of samples may occur and for that reason third factor, although sometimes with low eigenvalue, can help in better separation of clusters. Example of PCA for first three factors of five medicinal plants ground samples for particle sizes of <100 μm can be seen in Figure 5. 3D PCA is very useful in working with large sets of experimental data and is in intensive use nowadays.

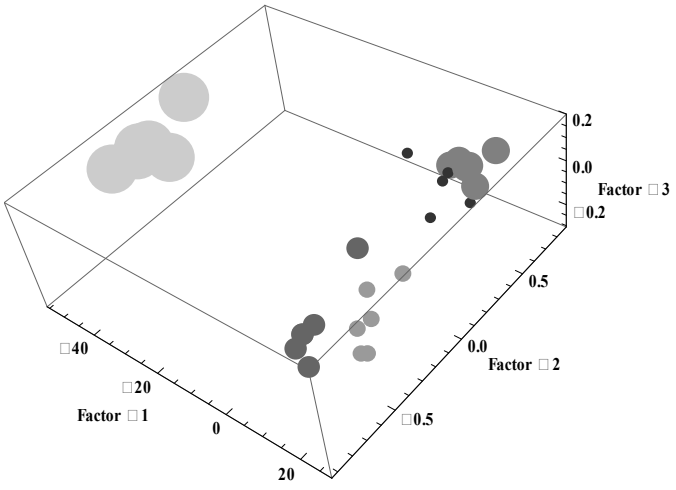


Fig. 5 Principal Component Analysis of five medicinal plants ground samples for particle sizes of <100 μm by first three factors (● sage, ● lavender, ● lemon balm, ● thyme, ● mint)

In Figure 5, better separation of size clusters was visible in comparison to 2D PCA. E.g. lemon balm, mint and sage, which were overlaping in 2D PCA are clearly separated with introduction of the third factor in 3D PCA. This confirmed the usefullnes of 3D PCA application when working with large datasets and its intensive use in data analysis.

Based on the NIR spectra of medical plants, the effectives of PCA application in distinguishing particle size (<100, 100, 250, 355, 500, 800 & 1000 μm) for the same plant, (Fig. 2., for 2 different medical plant), was confirmed. Then the same method (PCA) was applied for successful differentiation of different medical plants (Figure 4). The data matrix consisted of 796 points of the absorbance at NIR light, per one scan. Each medical plant extract was scanned at least 3

times. For the investigation of the effect of different particle sizes for one plant, the matrix consisted of 21 rows (different particle sizes and 3 repetitions) and 796 columns (absorbance at corresponding wavelengths starting from 904 to 1699 nm). In distinguishing different medical plants, the data matrix consisted of the same number of columns as previously stated, but the number of rows was the number of repeated scans (3x) multiplied with the number of investigated medical plants (5x), what resulted in total of 15 rows.

The next aim was to present the effectiveness of application of the same method on a much smaller data matrix, comprising of 7 different parameters measured for 6 medical plants (data matrix of 7 rows and 6 columns). In this case, PCA (Figure 6) was applied for determination of similarities and differences among six different medicinal plant extracts (marigold, sage, lemon balm, thyme, St. John's wort and lavender) depending on observed parameters (pH, conductivity, dry matter, total dissolved solids (TDS), total polyphenol content (TPC) and antioxidant capacity measured by DPPH method (AO) and Briggs-Rauscher method (BR-AO)). Water extracts were prepared at following conditions: $T = 40$ °C, $t = 90$ min and $rpm = 500$ min^{-1}.

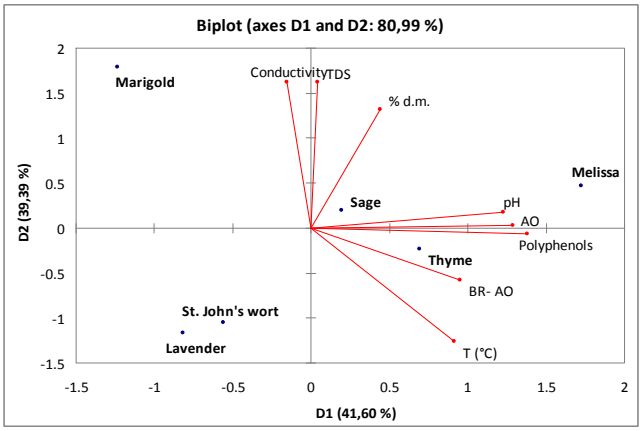


Fig. 6 PCA for water extracts of six medicinal plants (marigold, sage, lemon balm, thyme, St. John's wort and lavender) and seven observed parameters (pH, conductivity, dry matter, TDS, TCP,AO and BR-AO)

As presented in Fig. 6, water extracts of medicinal plants were distributed in all four quadrants. Water extract of marigold, which was situated in the second quadrant, had the highest conductivity, while extracts of St. John's wort and lavender, which were situated in the third quadrant, showed the lowest content of all measured parameters above the X-axis (conductivity, TDS, dry matter and pH). This confirmed the potential of NIRS in sample identification, in accordance with claims of other researchers [26, 27]. By using PCA, one can determine which parameters had significant influence on distribution of samples. Total polyphenols and antioxidant capacity measured by DPPH method and Briggs-Rauscher method had most influence based on first principal component, which explained 41.6 % of all the data interactions. Second principal component explained 39.39 % of variance where conductivity, TDS and dry matter were dominant. Totally 81 % of variance was explained for observed data (PC1 + PC2 = 80.99 %).

3.2.2 Effectiveness of use of Partial least squares regression

As mentioned previously, PLS regression is also a multivariate tool. It presents the projection on latent structures and its goal is to separate a set of dependent variables from a set of independent variables or predictors. The quality of the prediction obtained by the PLS regression model is evaluated with cross-validation techniques. PLS models for one medical plant (lavender) are presented, where the independent variables were the NIR spectra of lavender water extract at wavelengths 904-1699 nm, and the depended variables were observed experimental data for total dissolved solids (TDS), conductivity, dry matter, antioxidant activity (AOA) and the content of total polyphenols.

Considering that NIR spectroscopy is a non-destructive method, the aim of developing such PLS models was to predict the expected value or concentration of the parameter which was treated as a dependent variable in the model based on the NIR spectrum of the investigated sample. Such approach saves time, chemicals and workforce, which increases the efficiency of application of such models and the real sector.

Coefficient of determination (R^2) for an applicable prediction of a parameter based on the PLS model, should be higher than 0.9. Calculated coefficients for water extract of lavender are presented in Table 1. From five observed parameters, four of them were very well predicted based on the input data (NIR spectra): total dissolved solids (R^2 >0.98), conductivity of the extract (R^2 >0.99), dry matter (R^2 >0.96) and the content of total polyphenols (R^2 >0.98).

Table 1 Determination coefficients (R^2) for PLS regression models for different parameters of lavender extracts.

Plant	Parameter (depended variables of the PLS model(s))				
	Total dissolved solids	conductivity	dry matter	Antioxidant activity	content of total polyphenols
lavender	0.987	0.993	0.968	0.318	0.985

The antioxidant activity of lavender water extracts has not results in a successful model (R^2 < 0.9), which is an implication of a weak relation between the input and output variables.
The general outline of the PLS model equation in this example was:

$$y_i = b + a_1 \cdot x_1 + a_2 \cdot x_2 + a_3 \cdot x_3 + \cdots + a_n \cdot x_n \quad (1)$$

Where:
y_i = observed parameter (i = 1, for total dissolved substance (TDS); i = 2, for conductivity; i = 3, for dry matter content; i = 4, for antioxidant activity and i = 5, for concentration of total polyphenols)
xj = wavelength of the spectrum (j = 904 to 1699)

To present the effectiveness of "acceptable" models for prediction based on NIR spectra, the results were presented graphically in Fig. 7.

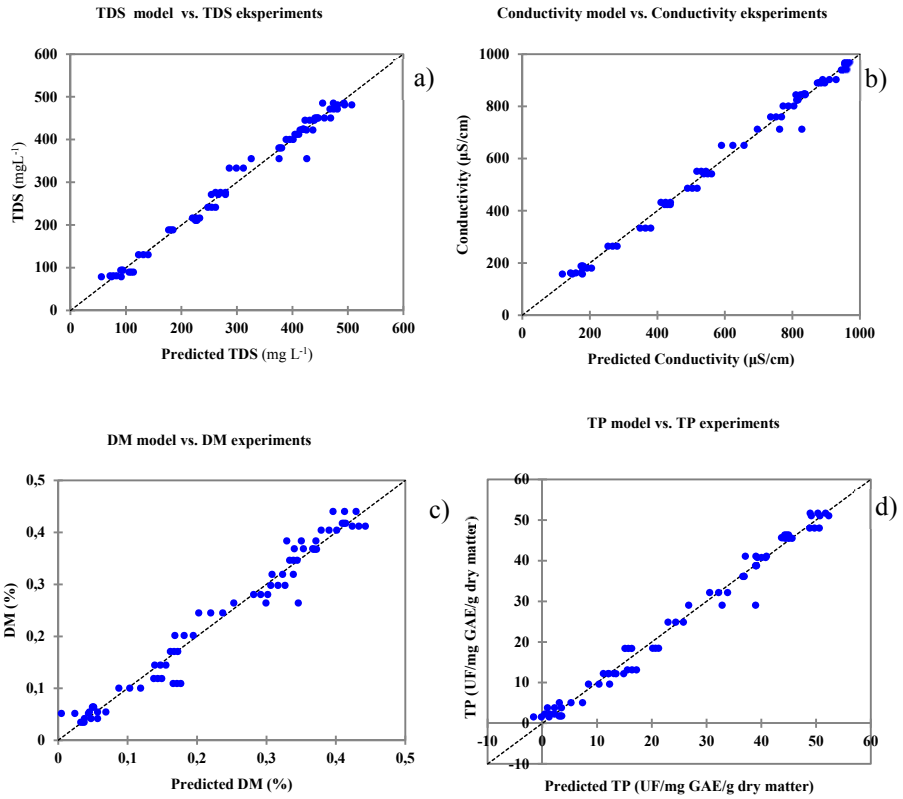


Fig. 7 Results of PLS regression for prediction of (a) TDS, (b) conductivity, (c) dry matter, (d) total polyphenols for water extracts of lavender at T = 60 °C, t = 90 min and rpm = 500 min^{-1}.

For the four models (TDS, conductivity, dry matter and total polyphenols) coefficient of determination and coefficient of correlation were obtained. The coefficient of determination represents the square of the coefficient of correlation and shows the percentage variation for y, which is explained by all the x variables together. For all the models coefficient of determination was higher than 0.96 which showed very good agreement between observer dependent and independent variables. Values for coefficient of determination for TDS, conductivity, dry matter and TP model were 0.987, 0.993, 0.968 and 0.985 respectively. This confirmed the adequacy of PLS models for prediction of physical and chemical properties of water extracts.

4. Conclusions

NIR spectroscopy combined with PCA as a chemometric method showed good potential for monitoring differeniation of medicinal plants and their grounded fractions in term of quality control. Results obtained by PCA and PLS regression models showed a very promising potential in monitoring physical and chemical properties of medicinal plant water extracts by NIR spectroscopy.

Acknowledgements This study is a contribution to the project „Application of microreactors in the analysis of antioxidant activity of medicinal plants (MICRO-AA)", project number HR.3.2.01-0069, which is funded by the European Social Fund (ESF) through the Human Resources Development.

References

[1] Nikolić, T., Rešetnik, I. Plant uses in Croatia. Phytologia Balcanica 2007; 13(2): 229–238.

[2] Wulandari, L., Retnaningtyas, Y., Nuri, Lukman, H. (2016) Analysis of flavonoid in medicinal plant extract using infrared spectroscopy and chemometrics. Journal of Analytical Methods in Chemistry 2016, Article ID 4696803, 6 pages, 2016.

[3] Damez, J.-L., Clerjon, S. Meat quality assessment using biophysical methods related to meat structure. Meat Science 2008; 80: 132–149.

[4] Xu, L., Zhou, Y.P., Tang, L.J., Wu, H.L., Jiang, J.H., Shen G.L., Yu, R.Q. (2008) Ensemble preprocessing of near-infrared (NIR) spectra for multivariate calibration. Analytica Chimica Acta 2008; 616(2): 138-143.

[5] Rinan, A., van den Berg, F., Balling Engelsen, S. Review of the most common pre-processing techniques for near-infrared spectra. Trends in Analytical Chemistry 2009; 28(10): 1201-1222.

[6] Jurinjak Tušek, A., Benković, M., Belščak Cvitanović, A., Valinger, D., Jurina, T., Gajdoš Kljusurić, J. Kinetics and thermodynamics of the solid-liquid extraction process of total polyphenols, antioxidants and extraction yield from Asteraceae plants. Industrial Crops and Products 2016; 91: 205-214.

[7] Saporta, G., Niang, N. Principal Component Analysis: Application to Statistical Process Control. In Data Analysis, (pp. 1-23): ISTE; 2010.

[8] Handa S.S., Khanuja S.P.S., Longo G. Rakesh D.D. Extraction technologies for medicinal and aromatic plants, International centre for science and high technology, Trieste; 2008.

[9] Lewis W.H., Elvin-Lewis M.P. Medicinal plants as a sources of new therapeutics. Ann. Mol. Bot. Gard. 1997; 82 (1995):16-24.

[10] Harrison, A.P., Bartels E.M. A modern appraisal of ancient Etruscan herbal practices. Am. J. Pharm.Toxicol. 2006;1(2):21-24.

[11] Benedek, B., Kopp, B., Melzig, M.F. Achillea millefolium L. s.l. – Is the anti-inflammatory activity mediated by protease inhibition? J. Ethnopharmacol. 2007; 113 (2): 312-317.

[12] Jebril A.O. A Screening Study on the Antimicrobial and Hepatoprotective Effects of Some Medicinal Plants in Libya. MSc. Thesis, Faculty of Science, Al-Fateh University, Tripoli, Libya; 2008.

[13] Ely, D.R., Thommes, M., Carvajal, M.T. Analysis of the effects of particle size and densification on NIR spectra Colloids and Surfaces. Physicochemical and Engineering Aspects 2008; 331 (1–2): 63–67.

[14] Barnes, R.J., Dhanoa, M.S., Lister, S.J. Standard normal variate transformation and de-trending of near-infrared diffuse reflectance spectra. Appl. Spectrosc. 1989; 43: 772–777.

[15] Papini, M. Study of the radiative properties of powdered and fibrous polymers. Vibrational Spectroscopy 1996; 11: 61–68.

[16] Pasikatan, M.C., Steele, J.L., Spillman, C.K., Haque, E. Near infrared reflectance spectroscopy for online particle size analysis of powders and ground materials. Journal of Near Infrared Spectroscopy 2001; 9: 153–164.

[17] Jurinjak Tušek, A., Benković, M., Belščak Cvitanović, A., Valinger, D., Jurina, T., Gajdoš Kljusurić, J. Kinetics and thermodynamics of the solid-liquid extraction process of total polyphenols, antioxidants and extraction yield from Asteraceae plants. Industrial Crops and Products 2016; 91: 205–214.

[18] Gajdoš Kljusurić, J., Djaković, S., Kruhak, I., Kovačević Ganić, K., Komes, D., Kurtanjek, Ž. Application of Briggs-Rauscher Reaction For Measurement of Antioxidant Capacity of Croatian Wines. Acta Alimentaria 2005; 34(4): 483-492.

[19] Valinger, D., Gajdoš Kljusurić, J., Kovačević, D., Kurtanjek, Želimir. Application of NIR spectroscopy for monitoring quality of surimi. Proceedings of 13th Ružička days, Today Science – Tomorrow industry 2011; 431-438.

[20] Jackson, J. E. A User's Guide to Principal Components. New York: John Wiley & Sons; 1991.

[21] Jolliffe, I. T. Principal Component Analysis (2 ed.). New York: Springer-Verlag New York; 2002.

[22] Saporta, G., & Niang, N. Principal Component Analysis: Application to Statistical Process Control. In Data Analysis, (pp. 1-23): ISTE; 2010.

[23] Abdi, H. PLS-Regression; Multivariate analysis. In M. Lewis-Beck, A. Bryman & T. Futing (Eds.), Encyclopedia for research methods for the social sciences., (pp. 792-795). Thousand Oaks (CA), USA: SAGE publications; 2003.

[24] Valinger, D., Benković, M., Jurina, T., Jurinjak Tušek, A., Belščak-Cvitanović, A., Gajdoš Kljusurić, J., Bauman, I. Use of NIR spectroscopy and 3D principal component analysis in divarication of different particle sizes of dried medicinal plants. Journal on Processing and Energy in Agriculture 2017; 21 (1): 17-22.

[25] Valinger, D., Benković, M., Gajdoš Kljusurić, J., Bauman, I., Kurtanjek, Ž., Application of NIR spectroscopy for monitoring different particle sizes of sucrose. Journal on processing and energy in agriculture 2011; 15 (3): 188-190.

[26] Gajdoš Kljusurić, J., Benković, M., Bauman, I. Classification and processing optimization of barley milk production using NIR spectroscopy, particle size, and total dissolved solids analysis. J. Chem. 2015; 201(5): 1-7.

[27] Budić-Leto, I., Gajdoš Kljusurić, J., Zdunić, G., Tomić-Potrebuješ, I., Banović, M., Kurtanjek, Ž., Lovrić, T. Usefulness of near infrared spectroscopy and chemometrics in screening of the quality of dessert wine Prošek. Croat. J. Food Sci. Technol. 2011; 3: 9-15.

Assess the physical and chemical characteristics and toxicity elements of the sludge of treatment plant: Minireview

F. Hallouz[1,2], **H. Karahacane**[1,2,3], **M. Meddi**[1] and **F. Sadi**[3,4]

[1] Engineering Laboratory of Water and Environment; ENSH, Blida – Algeria.
[2] Djilali BOUNAAMA University- Khemis Miliana, Ain Defla, Algeria
[3] Hassiba BEN BOUALI University - Chlef, Algeria
[4] Laboratory of local Natural Bio-resources, Faculty of Science, Hassiba BEN BOUALI-University Chlef, Algeria

This review is a survey on the latest and more recent applications of physical and chemical analyses to study different changes that plants undergo when using sludge as a fertilizer. Although sewage sludge is regarded today as waste by the new regulations, it has a real agronomic interest because of the presence of organic matter, nitrogen, phosphorus and a favorable carbon / nitrogen ratio. But to value the sludge must both respect the environment; look at the lowest cost possible and the most satisfactory technical solution.

This chapter covers recent trends in using sludge as natural fertilizer on field crops such as cereals and vegetable crops, then the sludge samples have been subjected to physical and chemical analyses such as: Hydrogen potential (pH), Electrical conductivity (EC), Organic matter (OM), total limestone / active Limestone, granulometry, humidity, cations exchange capacity (C.E.C)…

Keywords: physical and chemical; sludge; environment; fertilizer; pH

1. Introduction

The composition of the sludge varies considerably depending on the origin of the wastewater entering the treatment plant. Such wastewater may be of domestic, industrial or commercial origin; Or from urban stream. The composition of the sludge varies, depending on the respective share of each of these inputs.

According to Vergès (1984), the average value of sludge production is 50 grams of dry matter per person per day, representing a volume of 1 to 2 liters of liquid sludge per person per day.

Sewage treatment plant sludge (STEP) contains not only macro-elements (nitrogen, phosphorus) and organic matter generally used for soil remediation, but also contains 2 kinds of undesirable elements, because potentially dangerous for humans, animals or the environment: these are pathogens and chemical contaminants.

The main objective of the treatment of sludge in the treatment plant is to reduce the volume of the sludge in order to limit the quantities to be stored (or even spread) and to stabilize them in order to improve their physical characteristics (Improvement of their heap content) and to stop the biodegradation of which they are the center. Indeed, their high water content (99%) and the strong bacterial populations that are found there make it a culture broth favorable to the degradation of the fresh and fermentable organic matter that they contain, producing bad odors.

It has an impact on the growth and development of potato, tomatoes, and wheat. It has an objective:

- Assess the physical and chemical characteristics and toxicity of the sludge in treatment plant.

2. Conducting the analytical work

After removing the sludge from a sewage treatment plant and before starting the necessary analyzes, the steps to be taken are:

2.1 Sample drying

Sludge samples should be dried in a dry place at room temperature and then in an oven at 105 ° C for 24 hours.

Fig. 1 Sludge used (shot of Bouslimani and Benzara, 2015).

2.2 Sifting

The sludge must be screened with a 2-mm sieve.

2.3 Physico-chemical analyzes

The main analyzes carried out on our sludge are summarized in the table below

Table 1 Summary table of laboratory analysis measurements.

Parameters measured	Methods for measuring analyzes
Hydrogen potential (pH)	Dilute extract (1 / 2.5); pH meter
Electrical conductivity (CE)	Dilute extract (1/5); Conductimeter
Organic matter (M.O)	ANNE method
Total limestone / Active limestone	Calcimeter of BERNARD DROVINEAN
Granulometry	Method of STOCKES (by ROBINSON pipette)
Humidity	Drying in an oven at 105 ° C.
The cation exchange capacity (C.E.C)	Metson

2.3.1 Hydrogen potential (pH)

By definition, it is the unit of measurement of the concentration of hydrogen ions, which makes it possible to evaluate the acidity or the basicity of a medium; it defines the concentration of H + ions in the liquid phase of the soil [1]. Indeed, the pH varies from 0 to 14 and the neutrality is reached when the pH is equal to 7.

Table 2 pH Interpretation Scales: Extract 1 / 2.5 [2].

pH ≤5.5	Strongly acid
5.5<pH≤6.0	Frankly acid
6.0<pH≤6.5	Slightly acidic
6.5<pH≤7.0	Neutral
7.0<pH≤7.5	Slightly alkaline or slightly basic
pH>7.5	Alkaline or basic

There are several methods for measuring pH, the most accurate method for measuring the pH of a soil is to use an electrical "pH meter" which directly gives the pH value when immersing glass electrodes in a solution obtained in Mixing one part of soil sample with two parts of distilled water [3].

Sewage sludge should be mixed with lime (preferably quicklime rather than hydrated lime) in order to raise their pH to 12 and thus prevent the survival of potentially pathogenic microorganisms. This treatment of sewage sludge also makes it possible to increase the dryness of the sludge (dry matter content) because the quicklime reacts exothermically when in contact with the water contained in the sludge and steam is released.

A30% dryness is easily obtained whatever the dehydration equipment used. The mud / quicklime mixture should be as homogeneous and intimate as possible so that each lime grain is well reacted, allowing optimum yield [4].

According to [5], pH is a major factor in the retention of heavy metals because it controls all the processes affecting the behavior of these elements. The variation of the pH (natural in anthropic) seems to be the factor whose action has the most determining on the mobility of metals. Most trace metallic elements are more mobile under acidic conditions than under alkaline conditions [6].

The average pH for the sludge is in the range of 9.84 and 7.97, slightly alkaline to alkaline, so it is relatively favorable to all species.

2.3.2 Humidity

Field moisture corresponds to the moisture content of a soil sample at a given time; in particular at the time the sample was expressed as a percentage, moisture is obtained by weight difference in the sample after desiccation at 105 ° C for 48 hours.

Indeed, at the output of waste water treatment, the water content of the sludge is around 99% of the crude material. Reducing the masses to be handled by avoiding dust and improving quality are the issues involved in the recycling and recovery of sewage sludge. The processing proceeds by reducing the water content of sludge, in particular through drying.

In evaporating, the sludge loses all or part of their water and become solid.

The drying bed technique is practiced in open air on liquid sludge and combines natural evaporation and drainage of the free water through a filtering layer of sand and gravel.

This extensive system, depending on the weather conditions, produces solid sludge at 35 - 40% dryness [7].

2.3.3 Electric conductivity

This physicochemical measure gives us an idea of the concentration of the electrolytes in the soil solution on the one hand and the degree of salinization of the soil on the other hand.

The electrical conductivity with diluted extract (extract 1/5) is the salinity rate in the ratio of the quantity of soil to the quantity of water required for the preparation of the extract [8].

For the calibration of the apparatus: the Kcl (0.02N) is placed in the oven and the temperature is measured up to 25ºC.

Table 3 Classification of electrical conductivity [9].

Electrical conductivity (ms / cm)	Designation
<2.5	Unsalted
2.5-5	Low salt
5-10	Moderately salty
10-15	Dirty
15-20	Highly salty
20 to 27.5	Very strongly salted
>40	Hyper sale

For our sampled sludge, the electrical conductivity values range from 7.31 mS / cm to 7.08 mS / cm, so our sludge is part of the average salt class, Therefore, our sludge is part of the average salt class. On the other hand, excessive salinity of the sludge can cause damage to soils and plants.

2.3.4 Determination of Organic matter (ANNE method)

Organic matter is determined by means of organic carbon, taking as a fact that organic carbon accounts for 58% of the organic matter, using the ANNE method (1945). It consists of calculating:

$$\%C = \left(V_{control} - V_{sample}\right) \times 0.615/P \quad \text{.....(1)}$$

With:

P: soil weight is 1 gram.

$$MO = \%C \times 1.72 \quad \text{.....(2)}$$

Table 4 Classification of organic matter according to [10].

Type of soil	Organic material (OM) (%)
Very poor	< 14
Poor	14 ≤ OM < 20
Moderately poor	20 ≤ OM< 30
Rich	30 ≤ OM< 40
Very rich	OM ≥ 40

Generally, the sludge is rich in organic matter and contains nutrients (N, P, K and trace elements). These elements are useful for the good development of crops.

- Nitrogen (N) plays a major role in plant metabolism as a major component of proteins. It is essential for the growth of plants.
- Phosphorus (P) transports energy to the plant. It promotes the general growth of the plant, especially the root and the stems system. At the end of vegetation, it is stored in the storage organs to serve the development of future growth
- Potassium (K) enhances crop resistance to disease, drought and frost.

The trace elements (Copper, Magnesium, Zinc, etc.) are useful in reduced quantity to carry out all the chemical reactions that take place in the plant [11].

Indeed, according to AFNOR standards, the results of our sludge show that it is very rich in organic matter where the rates exceed 65%.

2.3.5 Determination of total limestone (BERNARD Calcemeters)

According to Aubert (1978), the evolution of limestone in our sludge must be monitored from the measurement of CO_2 evolution in BERNARD Calcimetre, and therefore by gasometry, this dosage is based on the characteristic reaction of calcium carbonate and Hydrochloric acid:

$$CaCO_3 + 2HCl \rightarrow CaCl_2 + H_2O + CO_2 \quad \text{.....(3)}$$

It is a question of comparing the volume of CO_2 released by the contact of HCL with a precise weight of sludge with that released by the contact of HCL with the pure and dry $CaCO_3$ in a known amount. The temperature and pressure conditions remain unchanged.

In addition, the presence of limestone plays an important role in ionic equilibria, particularly in pH values, and the limestone content is related to the nature of the substrate or to the different artificial inputs to correct the pH of the soil and to increase the buffer capacity of these studied soils [12].

2.3.6 Granulometry (ROBINSON pipette)

The purpose of granulometric analysis is to determine the texture of the soil by classifying the mineral particles constituting the soil sample by diameter category. The particles are separated by soil analyzes into three distinct classes: sand (2 to 0.05 mm), silt (0.05 to 0.02 mm) and clay (less than 0.02 mm) [13]. This allows us to know certain characteristics of the soil, such as the ability of roots to penetrate it, the capacity of the soil to retain water, or its vulnerability to compaction [14]; and therefore, the particle size distribution is carried out according to the international method which is based on the STOCKES law, which gives the settling velocity of a spherical particle in a liquid as a function of the diameter of the particle. This method uses the ROBINSON pipette, the texture is determined by a textural triangle. The removal of clays and fine silt was carried out using the Robinson pipette; the fine and coarse sands were recovered by sieving, the coarse silts were deducted by the difference [15].

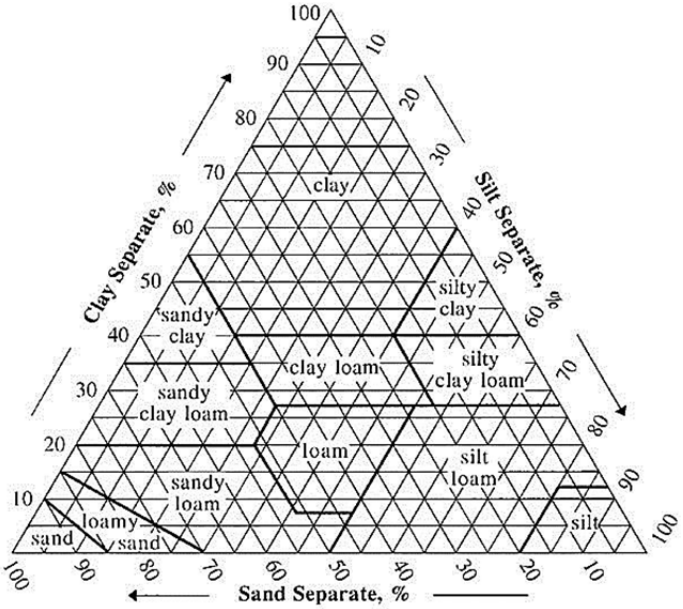


Fig. 2 Textural Triangle (American (USDA) triangle) [16].

The granulometry distribution of sludge is difficult and time-consuming to measure. The rare measurements carried out also show a strong dispersion around the mean diameter. On the other hand, if size is generally a favorable criterion, particles of large size can lead to problems with conveying (deposits) or abrasion (silica), especially in the case of centrifugation [17]**.**

According to the results, the sludge used in our experiment are made of: 20% clay, 5% silt, and 75% sand for our sludge.

If we compare these results to the US textural triangle (USA), we can conclude that our sludge has a Silty-sandy loam texture and according to [18], the texture obtained for sludge is middle class.

2.4 Nutrient Supply

However, the sludge contains some elements useful for plant growth, nitrogen, phosphorus, potassium and magnesium. The quantities vary from one sludge to another according to the origin and the mode of treatment [19]**.**

The crops most favored by the presence of nitrogen appear to be grass and corn, followed by cereals [20].

Phosphorus existing in less quantity than nitrogen.

As for the other three elements: potassium, calcium and magnesium, plants use them as oligo elements more than they use them as elements of development.

2.5 The mineral micropollutants

These are essentially what are called heavy metals, which have been extensively studied in the laboratory and in the field for their role in the development of irrigated crops by liquid or non-liquid sludge [21]**.**

Some of these elements are found naturally in the soil such as copper, iron, zinc ... and are essential to the growth of plants, while others are added by man and can have unfortunate consequences [22]**.**

2.6 The toxic elements

Indeed, these elements are not used by the plant for its development but can accumulate in it by accumulation [23]**.**

- **Aluminum**: is slightly soluble at pH close to neutrality and its content in plants is very variable. It becomes important for plants that grow in wet conditions. The toxicity of aluminum has not been demonstrated either for mammals or for humans [24]**.**
- **Arsenic**: As a salt, arsenic is as toxic to plants as it is to animals and man (arsenate or arsenite), but it seems that in organic form it is less dangerous. The inputs of this element come mainly from pesticides and very little sludge [25]**.**
- **Cadmium**: is brought into the soil by the atmosphere (rain), leaching of roads and sewage or sludge containing electroplating effluents. It is certainly the most studied micro pollutant in the case of sludge spreading because it is assimilated by plants and accumulated by mammals and humans (the quantity tolerable for humans is 0.3 ppm day). Cadmium is not very toxic to plants (especially leaves) and stored quantities depend on soil pH [26]**.**
- **Chromium**: is considered a toxic to humans and animals. Chromium is often found in sludge, but it seems to be transformed into the soil into sparingly soluble elements which cannot be easily assimilated. Tests of the addition of large quantities of chromium to the soil have shown that it has no disadvantages for plant yield [27]**.**
- **Mercury:** is an important toxicant for humans and animals, found in soil from insecticide application, rainwater and runoff, and sludge spreading. Floor. When the soil pH is above 6.5 mercury appears as a poorly soluble hydroxide or carbonate.
- Mercury can cause disturbances to the development of the plant, but the main danger is then its introduction into the human food chain because it is organic accumulated by the animals, but there does not seem to be any preferential accumulation in one part of the plant as has been seen for cadmium [28]**.**
- **Nickel:** present in industrial fumes and wastewater is found in soil by rainwater or sludge. The action of nickel alone on plants is known, it seems that a concentration of 1 p.p.m is enough to disrupt the growth of the plant [29]**.**
- **Lead:** The presence of lead in the soil leads to an accumulation on the surface layer. The origin of the lead is essentially atmospheric since a large part comes from the exhaust of cars. It enters the plant through the roots when it is in the soil and by the leaves when it is in the atmosphere but its presence does not seem to disrupt the development of the plant [30]**.**

In summary, it may indicate that some micro-pollutants are harmful to plants whether they promote withering, or they do lower the productivity and others are harmful to plants and consumers as they can be dangerous to human due to bioaccumulation. Their presence in sludge is rarely the only source but it is an additional risk that man brings to the level of agricultural production [31]**.**

3. Conclusion

A wastewater treatment plant exists to protect the surrounding environment including water resources. Sewage sludge has useful agronomic properties in the field of agriculture. Their use must take into account the nutritional needs of the plants without compromising the quality of the soils or that of the surface and ground waters. Indeed, some heavy metals in sewage sludge can be toxic to plants and humans.

References

[1] Dajoz, R.- Précis de pédologie. Ed. Bordas. Paris.1985; Dunod Université.p 331.
[2] ISO 10390:2005 – Qualité du sol – Détermination du pH.
[3] Duchaufour- Introduction à la science du sol – 2001 ; 6ème Ed de l'abréger de pédologie Dunod, 331p.
[4] Wiképidia. https://fr.wikipedia.org/wiki/Stabilisation_des_boues, 2017.
[5] Ramade F. Dictionnaire encyclopédique de l'Ecologie et des Sciences de l'Environnement. 1993, Paris. Edisciences International. 821 p.
[6] Alloway B. J. Heavy Metals in Soils. Editeur Springer Science & Business Media, 1995, 368 p.
[7] DomoSystem. Communiqué de presse Un nouvel Humitest pour mesurer la teneur en eau des boues d'épuration. Humidité et environnement, Paris, 2012.

[8] Samai, F. Le gisement calcaire de Djebel Safia : étude géologique, lithostratigraphique pétrographique et chimique. Mémoire d'ingénieur, 2002, 77 pages, Université de Annaba.
[9] ISO 11265:Janvier 1995 – Qualité du sol – Détermination de la conductivité électrique spécifique.
[10] NF ISO 14235. Qualite du sol – Dosage du carbone organique par oxydation sulfochimique. 1998, 11p.
[11] http://syprea.org/article/composition-boues.html
[12] Guerfi Z. Impact de l'utilisation des boues résiduaires sur les propriétés physico-chimiques des sols de la haute vallée de la Medjerda wilaya de Souk Ahras. Université de Badji-Mokhetar Annaba diplôme de magistère en écologie et environnement, 2012, 97p.
[13] Damay P. E., Maro D., Coppalle A., Lamaud E., Connan O., Hébert D., Talbaut M., Irvine M. Size-resolved eddy covariance measurements of fine particle vertical fluxes. Journal of Aerosol Science 40, 2009, 1050-1058.
[14] Calvet. - Le Sol propriétés et fonction: Tom I et II. Ed Dunod, 2003.
[15] Jones (K.P.), Mac Cave (I.N.), Patel (D.). – A computer-interfaced Sedigraph for modal size analysis P7f2fine-grained sediments. *Sedimentology,1988, 35* : 163-172.
[16] https://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/survey/?cid=nrcs142p2_054167. Natural Resources Conservation Service Soils. United States Department of Agriculture (consultation date 17/07/2017).
[17] Ademe**.** - Situation du recyclage agricole des boues d'épuration urbaines en Europe et dans divers autres pays du monde, 1999, Ademe édition, Paris, pp. 159.
[18] Henin, S. Cultural profile, physical state of the soil, its agricultural consequences. Masson (Ed.),1969, France, pp: 128.
[19] Pickford, J. - Sludge treatment and disposal. Fourth public health engineering conference, 1971.
[20] Debruck, J., Vomel, A.- Die Verwertung der Nahrstoff im Abwasserschlam Landwirtschftliche Forschung Dtsch, 1972, tome 17, N°1, P 39-60.
[21] Godin, P. - Sources de contamination et enjeux. - Séminaire Eléments traces et pollution des sols, 1982, Paris, 3 p.
[22] Miller R.W, Azzari A.S, Gardiner D.T. - Heavy metals in crops as affected by soil types and sewage sludge rates. - Commun., Soil Sci. 1995, Plant Anal., 26(5-6), p 703-711.
[23] Kirkham, M.B. - Disposal of Sludge on land: Effect on Soils, Pants, and Ground Water, 1974. Compost Science, P. 6-10.
[24] Leeper, G.W. - Managing the heavy metals on the land. Marcel Dekker, Inc, 1977.
[25] Gabarda, O.D. - Les boues de station d'épuration d'eaux usées urbaines: les filières alternatives à l'épandage. In Que faire des boues ? Une approche socio-économique du Club Environnement et Société. Ecrin, 2000, Paris. P. 58-60.
[26] Jones, R.L., Hinesly, T.D., Ziegler, E.L., Tyler, J.J. - Cadmium and Zinc contents of corn leaf and grain produced by sludge-amended soil. J Environ.Qual. 1975, vol. 4. N°4, P 509-514.
[27] Singh, B.R. - Trace element availability to plants in agricultural soils with, special emphasis on fertilizer inputs. - Environ. Rev. 2(2), 1994, p 133-146.
[28] Davies, B.E., White, H.M. - Trace elements in vegetables grown on soils contaminated by base metal mining. - J. Plant Nutr. 3, 1981, p 387-396.
[29] Aubert, G. - Méthodes d'analyse des sols. Marseille : 1978, G.R.D.P.191 p.
[30] Jorgensen, E. - An ecological model for heavy metal contamination of drops and ground water. Ecological Modelling, 1976, n°2, P 59-67.
[31] Hyde, H.C. - Utilization of wastewater sludge for agricultural soil enrichment.JWPCF, 1976, vol, 48, n°1, P 77-90.

Chitosan as alternative treatment to control postharvest losses of tropical and subtropical fruits

P. Gutierrez-Martinez[1,*] **A. Ramos-Guerrero**[1], **H. Cabanillas-Beltran**[1], **J. Romero-Islas**[1] and **A. Cruz-Hernandez**[2]

[1] Instituto Tecnológico de Tepic. Laboratorio Integral de Investigación en Alimentos. Av. Tecnológico 2595, Lagos de Country, Tepic,Nayarit. C.P. 63175. México.
[2] Escuela de Agronomía, Universidad De La Salle Bajío. Av Universidad 602. Col. Lomas del Campestre, León Gto. México. C.P. 37150.
* Corresponding author: email: pgutierrez@ittepic.edu.mx

Chitosan, a chitin derivative is a natural product obtained mainly from crustaceous. This natural, biodegradable and nontoxic compound has a broad potential in food, biotechnology, and agriculture industries. This review summarizes recently published literature describing experiments in which Chitosan have been tested on mycelia growth, germination of spores, enzyme activity and gene expression for different postharvest fungi and fruit tissues under in vitro and in vivo conditions. It has been found that chitosan controls postharvest infections by *Colletotrichum gloeosporioides, Alternaria alternata, Rhizopus stolonifer* and *Fusarium oxisporum*. Furthermore, the semipermeable characteristic of chitosan originates favourable physic-chemical changes on fruits metabolism, extending their storage life and the effects also include changes in enzymatic activity and genetic expression. Efforts on the evaluation of chitosan alone or in combination with some other compounds, must be done as alternative treatments on a wide scale to control postharvest losses of tropical and subtropical fruits.

Keywords: *Alternaria*; Antifungal activity; *Colletotrichum*; gene expression

1. Introduction

1.1 Post-harvest losses in tropical fruits

Globally, more than 63 million tons of tropical fruits are produced, producing almost 100% in developing countries [1]; [2]. Mexico is one of the main producers and exporters of tropical and subtropical fruits. However, these fruits are affected in the post-harvest stage by various pathogens, including *Colletotrichum gloeosporioides*, *Alternaria alternata*, *Fusarium*, *Rhizopus stolonifer* among others causing significant damage to the fruits, affecting the Post-harvest quality and preventing their commercialization, generating economic losses. Losses in developing countries range from 40 to 50 %[2, 3, 4, 5, 6]. Fungicides are the only method of control to date, with a high cost for the producer and the environment [6, 7, 8]. Chitosan is a natural, biodegradable and non-toxic biopolymer, various antifungal properties and as a resistance inducer, activating enzymes and genes that encode proteins related to the fruit defense system before the pathogen attack [9, 10]. It is necessary to explore other alternatives such as the use of compounds that occur naturally in plants and animals with fungicidal characteristics and with properties that induce defense mechanisms such as chitosan, a commercially viable alternative [11].

1.2 Post-harvest disease control systems for fruits

Due to the great losses of horticultural products in the post-harvest stage caused by pathogens, it is necessary to apply control methods to reduce these losses. Disease control has become difficult due to pathogen resistance to increasingly stringent fungicides and regulations. The great interest in human health and the environment has led to the study of alternative methods to the usual products that guarantee a similar or superior effectiveness to the synthetic fungicides, without the problems that they generate. The methods are classified according to their nature in: physical, including heat, such as Hot water treatment, steam, dry heat, forced air; Irradiation, low temperatures, modified and controlled hypobaric atmospheres, electrolyzed water and biological control, GRAS compounds such as volatile compounds, plant extracts, essential oils, Ethanol, Sodium bicarbonate, peptides and proteins. Finally Resistance Inducers: Focus on improving the individual potential of the host to respond to the attack of pathogens by activating defense mechanisms at the biochemical and molecular level. Products such as jasmonates, salicylic acid, and Chitosan. These control systems, individually are not 100% effective, when combined their effectiveness in controlling pathogens increases significantly [13, 14, 15, 16] .

Chitosan is a natural, biodegradable, non-toxic, bioactive polymer with fungicidal effects on the most important post-harvest pathogens affecting the quality of tropical and subtropical fruits as well as an increase in the losses of these products. In addition, chitosan is an inducer of defense mechanisms in plant tissues, including fruits [17, 18, 19]. In the post-harvest stage is one of the most promising products for the control of various fungi [20, 21, 22, 23].

2. Effects on Post-harvest Decay of fruits

Results obtained by López Mora et al. [21] in 2013 and Berumen et al. [11] in 2015 in mango Tommy Atkins, Coronado-Partida et al. [24] in 2017 in Jackfruit and Ramos-Guerrero et al. [25] in 2017 in Soursop fruits, and Xoca et al. [26] 2017 in Hass avocado, indicated that when applying chitosan at 1%, infection by *C. gloeosporioides, Fusarium oxisporum, Rhizopus stolonifer* was controlled (Fig.1). These results mentioned, chitosan individually or in some cases combined with another alternative control system, organic salts, hydrothermal treatment, salicylic acid, jasmonic acid, ethanol, etc. By combining chitosan with another system it is possible to use lower concentrations, which results in a more effective and economical treatment [27].

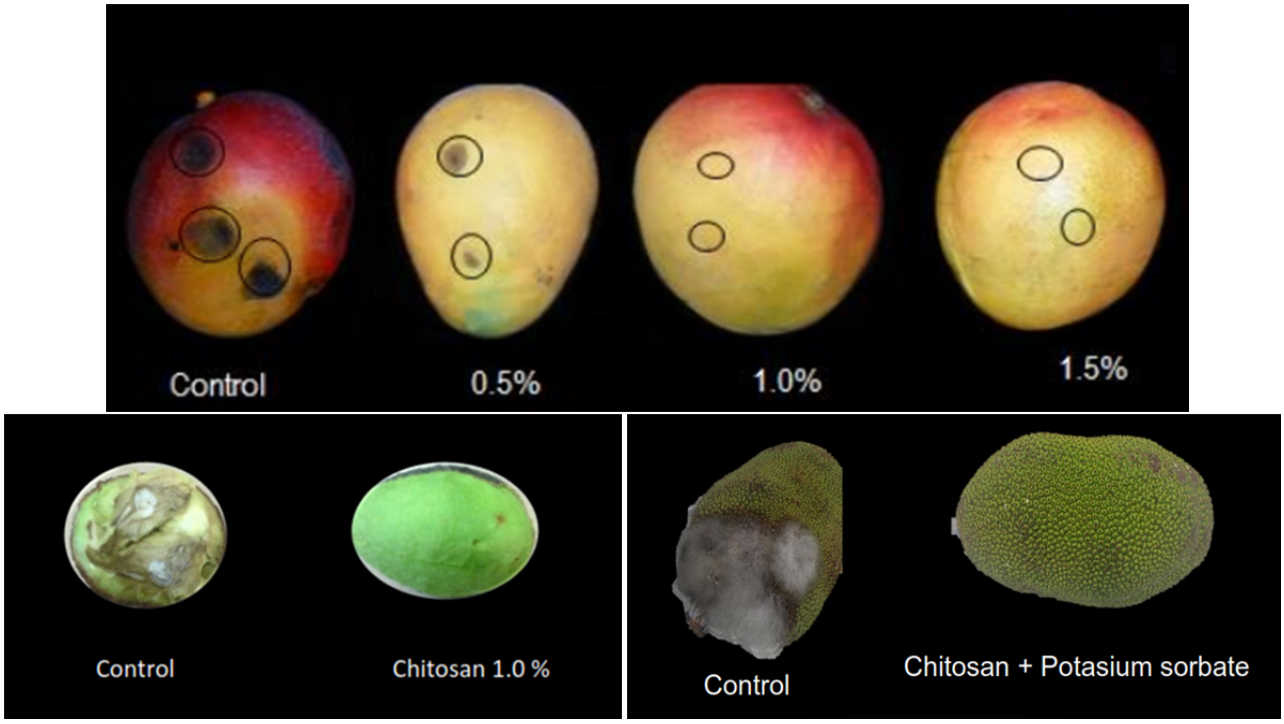


Fig. 1 Effect of chitosan and chitosan combine with Potasium sorbate on *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* postharvest control in mango. Avocado and Jackfruit 72 h after applying treatments, stored at 25°C.

2.1 Mechanisms of action.

Chitosan has an excellent fungicidal potential against the major fungi that cause damage to tropical fruits such as *Colletotrichum gloeosporioides* [28, 29, 30]. Some mechanisms of action proposed to explain fungicidal ability of chitosan, propose that the electrostatic interaction between the NH3 + groups of chitosan and the phosphoryl groups of the phospholipids present in the cell membrane of the fungi causes damage in this, causing an increase in permeability [31, 32, 33, 34]. In addition, it is possible that short chains of chitosan can pass through the wall and membrane and interact with the DNA and the RNA interfering with their function, losing functionality of the fungal structure[35, 36]. Its chelating effect could decrease the availability of some metals needed in enzymatic processes, inhibiting the process of the pathogenesis of fungi [37]. Alterations in the internal and external morphology of the conidia and mycelium caused by chitosan would provoke a biochemical-physiological stress [25].

2.2 Antimicrobial Properties

Before application of chitosan in fruits with the aim of reducing or inhibiting the growth of post-harvest fungi, it is necessary to determine the optimal concentrations that will decrease or stop mycelial growth as well as the concentration of chitosan capable of inhibiting the process of spore germination. López-Mora et al. [21] 2013, applied chitosan to the *A. alternata* fungus, isolated from mango fruits and observed a gradual control of the growth and development of the fungus as the concentration of chitosan increased (Figures 2 and 3)(Table 1). Similar results were obtained in the *Colletotrichum*-Mango interaction system [24], *Colletotrichum-Fusarium*-Banana [29], *Rhizopus*-Jackfruit [38] *Colletotrichum-Rhizopus*-Soursop [25], where chitosan control growth, inhibited germination of spores and altered morphology of conidia

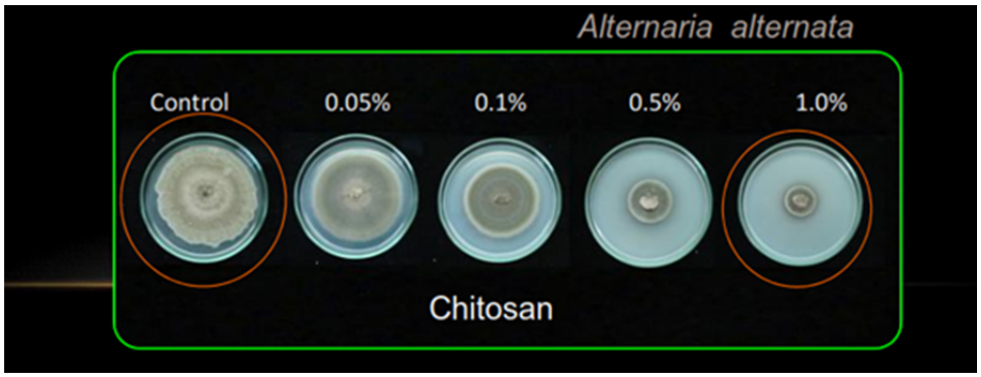


Fig. 2 Effect of chitosan on mycelial growth of *A. alternata*.

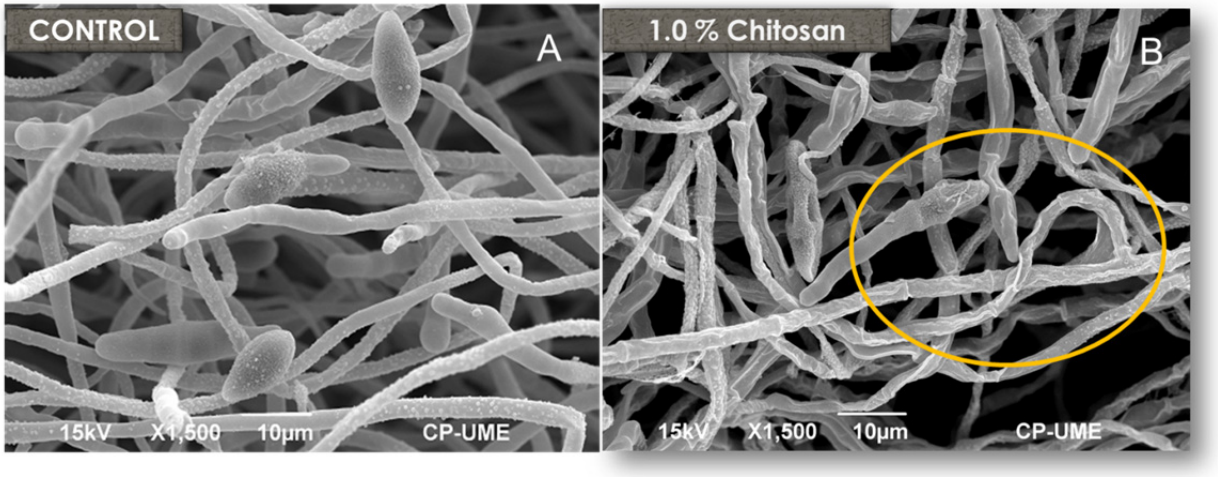


Fig. 3 Effect of Chitosan on *C. gloeosporioides* conidium. Control (A). Chitosan 1.0%B). Scanning Electron Microscopy.

Table 1 In vitro development of two isolates of Colletotrichum chitosan-treated at different concentrations and incubated at 20 ± 2ºC.

Chitosan Concentration (%)	Soursop			Mango		
	Rate of growth (mm día^{-1})	Sporulation	Germination (%)$^{+}$	Rate of growth (mm día^{-1})	Sporulation	Germination (%)$^{+}$
0.5	3.4^{c}	2.2 x 10^{6c}	20^{b}	5.4^{b}	3.5 x 10^{5c}	13^{b}
1.0	1.3^{b}	1.3 x 10^{6b}	0a	5.0^{b}	1.5 x 10^{5b}	0^{a}
1.5	0.9^{a}	1.2 x 10^{6a}	0a	4.5^{a}	1.0 x 10^{5a}	0^{a}
Control	5.8^{d}	3.3 x 10^{6d}	100^{c}	5.8^{c}	7.5 x 10^{5d}	100^{c}

Means followed by the same letter are not significantly different (≤ 0.05) as determined by Tukey's multiple test. Rate of growth after 12 days of incubation. Sporulation and germination after 8 h incubation.

2.3 Inducing properties

The use of chitosan as an inducer of resistance may be significant if one considers the systemic and persistent nature of defense proteins in plant tissues in response to the presence of chitosan, which may be important in delaying the resumption of an infection Latent tissue that typically begins to activate when tissue resistance declines [39,17, 20]. One of the current trends in the control of post-harvest diseases is to stimulate the fruit and vegetable product to reactivate its own defense mechanisms [4] [15]. Zhang et al. [18] in 2011, indicated that as an exogenous inducer the chitosan can activate resistance in the host by increasing the activities of various defense-related enzymes, such as chitinase and β-1,3- Glucanase in oranges, Berumen et al. [11] in 2013, reported that it was possible to induce the activity of PFO and POD enzymes, important in the plant defense system, being the concentration of 1% chitosan that obtained Better results being able to induce more the enzymatic activity of both enzymes in mango (Figure 4).

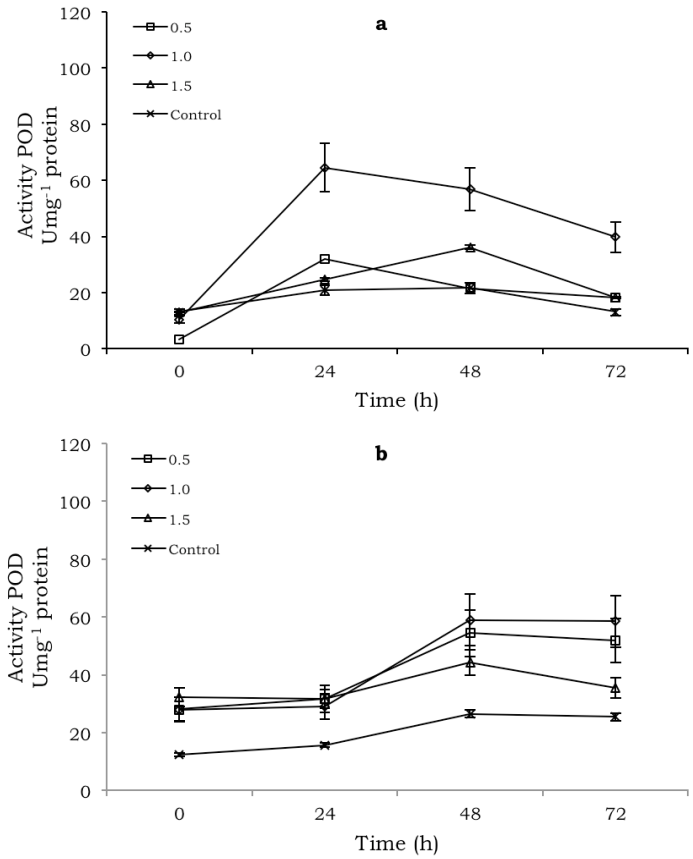


Fig. 4 Effect of chitosan at different concentrations on POD activity in mango fruits cv. 'Tommy Atkins': a) uninoculated and b) inoculated with *Colletotrichum* sp. Bars indicate mean standard error of 5 observations per treatments.

The information that exists in relation to the induction by the chitosan is mainly related to the analysis of the enzymatic activity of proteins related to the defense to pathogens As for the information generated in tropical fruits, considering the inductive capacity of the chitosan, is scarce (Mango, soursop, banana, jackfruit) [11, 25, 29, 38] and even more when referring to the use of molecular biology to evaluate the effect of chitosan at the genetic level, -PCR of the genes involved in the defense of the plant system (fruit or plant). Few reports exist on the evaluation of the effect of chitosan on the pathogen, at the spore or mycelial level [30], considering the expression of genes, in fungi as important from the point of view of post-harvest as is the genus *Colletotrichum*, agent Pathogen that infects and causes large losses in tropical fruits such as mango, banana, soursop, avocado, papaya, etc. Berumen in 2013 [11] reported that when applying 1% chitosan films to mango fruits, it was observed that there was an increase In early genetic expression of the *C. gloeosporioides* infection in mango fruits, suggesting that chitosan acts from the contact with the host, through a process of signal transduction, thus achieving the induction and increase of the Gene expression of both enzymes (Polyphenol oxidase (PPO) and Peroxidase (POD), generating the activation of the fruit defense mechanisms [11].

Hernández-Ibañez [29] evaluated the genetic expression of NPR1 (a gene related to defense systems) by applying 1.5% of chitosan in banana fruits inoculated with *C. gloeosporioides*, observing a high genetic expression in the early Pathogen-fruit-chitosan interaction. Ochoa-Jiménez [30] in 2012, analyzed the expression of the gene encoding polygalacturonase (PG) in spores of *C. gloeosporioides*, isolated from banana fruits, reporting a decrease in the expression of the polygalacturonase (PG) gene, , As a consequence of the application of chitosan to 1%, which has as consequence the alteration of spore germination.

Xoca et al. [40] in 2017 indicate that the chitosan-avocado-*Colletotrichum* interaction transcriptomic analysis proposes an effect of chitosan at the level of induction of metabolic pathways to eliminate the pathogen *Colletotrichum*. In conclusion, Chitosan has shown great potential as a natural substance, biodegradable, biocompatible without toxicity or side effects with antifungal activities with a direct effect on the growth and development of the fungus at the level of conidium and mycelium.

A very important effect of chitosan is the induction of defense mechanisms at the fruit level. The information generated by transcriptome studies in chitosan-pathogen interaction systems indicates the effect of chitosan on metabolic pathways important for the defense of the fruit against the attack of the pathogen and in the fungus, resulting in morphological and biochemical alterations that Inhibit the growth and germination of the pathogen. Surely with the new technologies omics will have more idea of the mechanism of action of chitosan in interaction with the pathogen and in the interaction of the fruit in the post-harvest stage. It is necessary to perform tests at the level of tropical fruit

packers and thus to have the complete picture from the in vitro, in situ and packing tests that will allow making formulations and to market products based on chitosan, as bio-fungicide.

References

[1] FAO. Definitional framework of food loss. Working Paper. Rome 2014.
[2] Vishal S, Hedayetullah M, Parveen Z, Jagamohan M. Postharvest Technology of Fruits and Vegetables: An Overview. J Postharvest Tech. 2014; 02(02):124-135.
[3] FAO. Global food losses and food waste – Extent, causes and prevention. Rome; 2011
[4] Bautista-Baños S, Romanazzi G, Jiménez-Aparicio A. Chitosan in the Preservation of Agricultural Commodities. USA: Academic Press/Elsevier; 2016.
[5] Droby S, Wisniewski M, Benkeblia N. Fruit and strategies for decay control. In: Postharvest technologies to maintain the quality of tropical and subtropical fruits. Ed E. M. Yahia and J. De Jesus Ornelas-Paz A. Elansari. Fundamental issues. Woodhead publishing limited. UK 2011; 1:194-224.
[6] SIACON-SAGARPA. Subsistema de Información Agrícola. SAGARPA. Consulta de Indicadores de Producción Nacional y Márgenes de Comercialización de frutos tropicales en México; 2011.
[7] Rondón O, Sanabría D, Albarracín N, Rondón A. Respuesta in vitro a la acción de fungicidas para el control de antracnosis, (*Colletotrichum gloeosporioides* penz), en frutos de mango. Agron Trop 2006; 56(2):219-235.
[8] Ploetz RC. Antracnosis en mango: Manejo de la enfermedad más importante en pre y postcosecha. Universidad de la Florida, USA. Dept of Phytopathology 2009;1:1-11.
[9] Mari M, Di Francesco A, Bertolini P. Control of fruit postharvest diseases: old and innovative approaches. Stewart Postharv Rev 2014; 1:1.
[10] Badawy MEI, Rabea EI. Potential of the biopolymer chitosan with different molecular weights to control postharvest gray mold of tomato fruit. Postharvest Biol Technol 2009; 51(1):110-117.
[11] Berúmen-Varela,G, Coronado-Partida L, Ochoa-Jiménez, V, Chacón- López, M, Gutiérrez-Martínez P. Effect of chitosan on the induction of disease resistance against *Colletotrichum sp*. in mango (*Mangifera indica L.*) cv Tommy Atkins. Investigación y Ciencia. 2015; Vol. 66. 16-21 pp.
[12] Mohamed C, Polidori J, Montel D, Loiseau G, Noêlle D. Preservation of mango quality by using functional chitosan-lactoperoxidase systems coatings. Postharvest Biol Technol. 2015; 101: 10-14.
[13] Feliziani E, Lichter A, , Smilanick J, Ippolito I. Disinfecting agents for controlling fruit and vegetable diseases after harvest, Postharvest Biol. Technol. 2016. http://dx.doi.org/10.1016/j.postharvbio.2016.04.016
[14] Usall J, Ippolito A, Sisquella M, Neri F. Physical treatments to control postharvest diseases of fresh fruits and vegetables. Postharvest Biol Technol. 2016; 122(2016)30–40.
[15] Romanazzi G, Sanzani S, Bi Y, Tian S, Gutiérrez-Martínez P, Alkan N. Induced resistance to control postharvest decay of fruit and vegetables. Postharvest Biol Technol. 2016; Vol. 122, December, Pages 82–94.
[16] Palou L, Asgar A, Fallik F, Romanazzi R. GRAS, plant- and animal-derived compounds as alternatives to conventional fungicides for the control of postharvest diseases of fresh horticultural produce. Postharvest Biol Technol. 2016; 122 41–52
[17] Terry A, Joyce DC. Elicitors of induced disease resistance in postharvest horticultural crops: A brief review. Postharvest Biol Technol; 2004; 32:1–13.
[18] Zhang H, Li R, Liu W. Effects of chitin and its derivative chitosan on postharvest decay of fruits: A Review. Int J Mol Sci. 2011; 12:917-934.
[19] Gutiérrez-Martínez P, Bautista-Baños S, Barrera-Necha L. Uso potencial de extractos vegetales, aceites esenciales y quitosano para reducir el daño causado por hongos postcosecha en productos hortofrutícolas. In: Ramírez de León JA. Ed. Avances en ciencia y tecnología alimentaria en México .2012;1:485-495.
[20] El-Hadrami A, Lorne A, El-Hadrami I, Dayf F. Chitosan in Plant Protection. Mar Drugs. 2010; 8(4):968-987.
[21] López-Mora L, Gutiérrez-Martínez P, Bautista-Baños S, Jiménez-García, L, Zavaleta- Mancera H. Evaluation of antifungal activity of chitosan in *Alternaria alternata* and in the quality of "tommy atkins" mango during storage. Rev Chapingo Ser Hortic. 2013;19(3):1-15.
[22] Liu J, Tian S, Meng X, Xu Y. Effects of chitosan on control of postharvest diseases and physiological responses of tomato fruit. Postharvest Biol Technol. 2007; 44(3):300-306.
[23] Bem S, Ardi R, Pinto R, Aki C, Fallik E. Controlling gray mould caused by *Botrytis cinerea* in cucumber plants by means of chitosan. Crop Prot 2003; 22:285-290.
[24] Coronado-Partida L, Gutierrez-Martinez P. Effect of Chitosan in Colletotrichum gloeosporioides. Int J Food Sci Technol. Forthcoming. 2017.
[25] Ramos-Guerrero A, Romanazzi, Gutiérrez-Martínez, P. Effects of chitosan in the control of postharvest decay of soursop (*Annona muricata*) fruit. Postharvest Biol and Technol. Forthcoming.2017
[26] Xoca-Orozco LÁ, Gutiérrez-Martínez P. Effect of chitosan on growth of Colletotrichum gloeosporioides and quality of avocado fruit c.v. Hass (*Persea americana*). Int J Food Sci Technol. Forthcoming. 2017.
[27] Miramontes-Serrano E, Hallswort J, Moscoso-Ramirez P, Gutierrez-Martinez P. 2017. Thermal stress and chitosan as an alternative control system against *Colletotrichum gloeosporioides* isolated from mango (*Mangifera indica L*) c.v.Tommy. Postharvest Biol and Technol; Forthcoming.2017
[28] Gutiérrez-Martínez P, Bautista-Baños S, Berumen-Varela G, Ramos-Guerrero A, Hernández-Ibañez AM. In vitro response of *Colletotrichum* to chitosan. Effect on incidence and quality on tropical fruit. Enzymatic expression in mango. Acta Agron. 2017. ; 66 (2) p 282-289.
[29] Hernández-Ibáñez AM, Bautista-Baños S, Gutiérrez-Martínez P. Potential of chitosan for controlling crown rot disease of banana (*Musa acuminata*) fruit cv. 'Enano gigante'. advances in science, Biotechnol Safety Foods-AMECA 2013; 19-136.

[30] Ochoa-Jiménez A, Berumen-Varela G, Chacón-López MA, Gutiérrez-Martinez P. Analysis of Some Mechanisms of Inhibition by Chitosan in Cell Germination of *Colletotrichum* sp isolated from Banana Fruits (*Musa sapientum*). Advances in Science, Biotechnol Safety Foods-AMECA 2015; 321-326.

[31] Fernández C, Ausar S, Badini R, Castagna L, Bianco I, Beltramo D. An FTIR spectroscopy study of the interaction between αscasein-bound phosphoryl groups and chitosan. Int Dairy J. 2003; 13(11):897-901.

[32] Guerra-Sánchez MG, Vega-Pérez J, Velázquez-del Valle MG, Hernández-Lauzardo AN. Antifungal activity and release of compounds on *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill. by effect of chitosan with different molecular weights. Pestic Biochem Phys 2009; 93(1):18-22.

[33] Palma-Guerrero J, Lopez-Jimenez J, Pérez-Berna A, Huang I, Jansson H, Salinas J, Villalaín J, Read N, Lopez-Llorca L. Membrane fluidity determines sensitivity of filamentous fungi to chitosan. Mol Microbiol .2010; 75:1021-1032.

[34] García-Rincón J, Vega-Pérez J, Guerra-Sánchez M, Hernández-Lauzardo A, Peña-Díaz A, Velázquez-Del Valle M. Effect of chitosan on growth and plasma membrane properties of *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. Pestic Biochem Phys; 2010; 97 (3):275-278.

[35] Xu J, Zhao X, Han, X, Du Y. Antifungal activity of oligochitosan against *Phytophthora capsici* and other plant pathogenic fungi in vitro. Pestic Biochem Phys. 2007a; 87(3):220-228.

[36] Xu J, Zhao X, Wang X, Zhao Z, Du Y. Oligochitosan inhibits *Phytophtora capsici* by penetrating the cell membrane and putative binding to intracellular target. Pestic Biochem Phys. 2007b; 88(2):167-175.

[37] Bueter C, Specht C, Pearls L. Innate Sensing of Chitin and Chitosan. PLoS Pathog. 2013;9(1):1-3.

[38] Coronado Partida, L, Amezcua-Vega C, Gutiérrez-Martínez P. Effect of chitosan and GRAS substances on post-harvest control of *Rhizopus sp* and the induction of the enzymatic activity of Peroxidase (POD) and Polyphenoloxidase (PFO) in jackfruit (*Artocarpus heterophyllus L.*). Crop protection. Forthcoming.2017.

[39] Wilson CL, El-Gahouth A, Chaluts E, Droby S, Stevens C, Lu JL, Khan V, Arul J. Potential of induced resistance to control postharvest diseases of fruits and vegetables. Plant Dis. 1994; 78:837-844.

[40] Xoca-Orozco LÁ, Cuellar-Torres EA, González-Morales S, Gutiérrez-Martínez P, López-García U, Herrera-Estrella L, Vega-Arreguín J, Chacón-López A. Transcriptomic Analysis of Avocado Hass (*Persea americana* Mill) in the Interaction System Fruit-Chitosan-*Colletotrichum*.Front. Plant Sci. 2017; 8:956

[41] Gutiérrez-Martínez P, Chacón-López AM, Xoca-Orozco LA, Ramos-Guerrero A, Velázquez-Estrada RM, Aguilera-Aguirre S. Chitosan and changes in gene expression during fruit-pathogen interaction at postharvest stage. In: Chitosan in the preservation of Agricultural products. Ed. Bautista-Baños, S., Romanazzi, G.F and Jimenez-Aparicio, A. Academic Press (Elsevier). .2016; 380 pp.

Contamination by fusariotoxins in Zea Mays L. (maize) for human consumption

S.D. Peña Betancourt[1] and **S. De Saeger**[2]

[1] Departament of Agricultural and Animal Production. UAM-X. Calzada del Hueso 1100, Mexico
[2] Laboratory of foods analysis. Faculty of Pharmaceutical Sciences Ghent University, Belgium

Abstract. In Mexico, mycotoxin contamination had been studied in maize grain and its products destined for human consumption, particularly aflatoxins and fumonisins [1]. The national regulation establishes a level maximum of 20 µg kg for unprocessed maize [2]. We have investigated in maize the physical and chemical composition, resistance to *Fusarium sp* and mycotoxins contamination. The results showed that *Fusarium sp* is a common fungus in agricultural soil in the central states of the country, whose species are *Fusarium proliferatum*, *F. roseum, F. oxysporum* and *F. poae*. Fumonisins, Fusarenone X, Enniatin A and mycophenolic acid that are the most prevalent fusariotoxins. The detected levels of fumonisins and mycophenolic acid are low but in a high frequency. It is concluded that the maize genotypes evaluated are susceptible to fungus *Fusarium sp* and fusariotoxins, under the environmental conditions of central regions in Mexico. The aflatoxin contamination was presented in both genotypes, yellow and white. It is important to consider fusariotoxins in a commercial maize regulation for human consumption; due to the long periods and high consumption of maize it is a risk to hepatotoxicity and cancer development.

Keywords: fusariotoxins; maize

1. Introduction

Mexico has been considered the origin of maize, crops are grown in 7 million of ha, principally in tropical and subtropical zones, being the principal varieties white and yellow, with a national production about 12 million tons per year [1, 2]. The corn is the base of 300 hundred by-products as fructosed honey, nixtamalized fluors, tortillas and tortilla chips for human consumption. It is estimated that the annual consumption of corn is 300 kg per person, in which more than 500g per day as tortillas; however, an excessive consumption may contribute to obesity in children [3]. In the field, maize crop can be infected by fungus pathogens, as *Fusarium sp*, which synthetizes fusariotoxins, due to water stress conditions, nitrogen excess, acidity in soil, lack of oxygen. Grain storage for long periods coupled with poor hygiene and high temperatures contributes to *Aspergillus sp*, infestation, causing a fungal mycoflore magnification and mycotoxin contamination [4, 5].

In Mexico aflatoxins research in maize began with an alert in 1989 in the north of the country, where aflatoxins were detected at levels not suitable for human consumption. Ten years later, in 1999, 95% of white corn still had aflatoxins; In 2013, it was mentioned a frequency of 56%, in allowed average levels of 1 to 18 µg kg, so it has been regulated the presence of total aflatoxins in a maximum permitted level of 20 µg kg [6].

Aspergillus flavus, Aspergillus parasiticus, Aspergillus nomius, are microscopic saprophytic fungi that develops at a relative humidity between 70 and 90% and over a wide range of temperatures, ranging from 0 ˚C to 45 ˚C, so they are able to infect the maize plant from the field and continue during harvesting and storage. These pathogenic fungi have been detected in the agricultural soils of central Mexico (Guanajuato, Colima, Nayarit), which can synthesize aflatoxins [7]; they are chemical substances, product of the secondary fungus metabolism, which are produced from simple intermediates of primary metabolism such as acetate, malonate and certain amino acids, which possess great thermal stability, that confers dangerous physical and chemical properties to human health [8]. The International Agency for Research on Cancer considered aflatoxins as substances with a high carcinogenic, mutagenic and teratogenic capacity [9]. In Asian and African populations, aflatoxin B1 has been epidemiologically related to liver cancer. Aflatoxin M1, is genotoxic, mutagenic, teratogenic and carcinogenic; it is found in the milk of cattle that have been fed with aflatoxin contaminated feed [10].

F. verticillioides, a saprophytic fungus in agricultural soils, with high global prevalence, and great virulence, causes a systemic infection known as fusariosis, which is a devastating disease for crops It is capable to synthesize fusariotoxins, among which, fumonisins that are chemically polyketides, are toxic substances capable of disturbing phospholipids metabolism, which are found in many tissues mainly in nerve tissue and fat tissue, whose function is cell protection and cell apoptosis signaling. Fumonisins prevent the formation of the enzyme sphinganine N-acyl transferase and de novo synthesis of sphingolipids, triggering a buildup of sphinganine. In animals the lack of phospholipids causes a demyelination of neurons, occurring necrosis of nerve cells and polioencephalomalacia [11]. In humans the consumption of fumonisin contaminated maize has been epidemiologically related to neural tube defects and esophageal cancer [12], due to the lipid peroxidation induced on the cell membrane causing cell death and inhibition of macromolecule synthesis, so the International Cancer Research Center classified them as possible human carcinogens for unprocessed maize it is recommended a maximum content of 1.0 mg kg, and in corn flours for consumption of 750

mg kg. It has been documented the contamination by fumonisins in corn flour and in tortillas in amounts of 1.80 mg/kg, even fumonisin metabolites (FB1H, FB2H, FB3H), of greater toxicity have also been identified. The Food Codex Commission established the daily intake of 2 ng g for fumonisins [13, 14].

The analytical method used to isolate and identify mycotoxins is High Performance Liquid Chromatography (HPLC), due to its high sensitivity of 5 μg/g [15], the lateral flow immunoassay (IFL) is a green technology and environmentally friendly. Liquid chromatography tandem mass HPLC/MS/MS is now used [16]. The Laboratory of Toxicology of UAM-X University, has works with diferents genotypes of maize from Mexico City, Morelos and Hidalgo, zone with sutropical and tropical climate. The mycotoxins identification was made with a cromatographic identification (HPTLC, HPLC-FD) and immunochromatographic test [17].

2. Sampling, extraction and analysis

During 2013-2014, twenty nine samples were evaluated; twenty five samples of maize (sixteen white maize and nine yellow maize) were obtained from Zacatepec, in the state of Morelos, located between the coordinates of 18 ° 37 'and 18 41' altitude N and 99 ° 10 'and 99 ° 14' longitude O; at a height between 900 and 1200 m above sea level, with a minimum temperature of 24°C and maximum of 40°C, with an average annual precipitation of 82 mm.

A random AFB_1 monitoring was performed on eight maize samples selected for their greater commercialization in the state of Morelos. Eight samples were processed using the High Resolution Liquid Chromatography (HPLC) technique, with fluorescence detector (FD). An isocratic pump brand Varian, model Polaris was used. Column of C18, size of 150 mm x 4.6 mm and of 5 microns of diameter. 50 g of pulverized flour was weighed and mixed with 100 mL of 80% methanol, leaving on mechanical agitation for 30 min. The extract was filtered through Waltham paper (No. 41); 10 mL was extracted and passed through a C18 silica column, from which 25 μL were taken and injected in triplicate to the chromatograph. The mobile phase was composed of water, methanol and acetonitrile (60:20:20 v/v/v). The fluorescence detector at a excitation length (λ 360 nm) and emission (λ 440 nm), with a mobile phase flow of 1 mL per minute. The calibration curve ($y = 3128699.71x + 218056.30$) showed a significant linearity ($p < 0.05$) in a range of 0.10; 0.25; 0.5; and 1 μg / mL with a regression coefficient of 0.99. The limits of detection and quantification were 0.241 and 0.43 μg/kg, respectively. The recovery (accuracy) was 87%. In addition, two samples were collected from Hidalgo State, and the rest directly from maize producers in Mexico City were analyzed by UHPLC/MS/MS [16].

Twenty five samples were analyzed for fumonisins in a lateral flow immunoassay technique, starting from 5 g of the sample and an extraction with 50 mL of the buffer solution (PBS), the suspension was mechanically mixed for two minutes and the supernatant was placed in the immunological strip where the Ag-Ac reaction concludes, by identifying two bands as positive test. The quantification was done by inserting the strip into reader [17].

3. Results and Discussion

The presence of pathogenic fungal flora in white maize adapted to areas with a tropical and subtropical climate indicates the susceptibility of genotype maize to *Fusarium sp*, as *Fusarium proliferatum*, *F. roseum, F. oxysporum* and *F. poae* and other fungus, in low incidence, as *Alternaria sp* or *Penicillium sp*, in samples from Hidalgo. The results of fumonisins contamination in twenty nine maize samples are presented in Table 1, where it is observed that all the samples presented fumonisins contamination. The white maize with levels of 0.37 mg kg, with a minimum value of 0.23 mg kg and a maximum of 1.20 mg kg, in yellow maize with an average level of 0.51 mg kg, with a minimum of 0.23 mg kg and a maximum of 1.7 mg kg; being the P 2844 and P4032 genotypes of the greater contamination. Fumonisin contamination has been linked to esophageal cancer and neural tube defects [18].

The aflatoxins contamination was found in both genotypes, yellow with a mean of 8.16 μg kg and 6.7 μg kg for white maize. The NB genotype was the most contaminated from the state of Morelos; all within the national regulation for aflatoxins, however outside the European regulation, which is 5 μg kg. On the other hand, observing the co-contamination of both toxins, it suggests a synergistic effect between the hepatotoxic activity of aflatoxin and the cancer promoting effect of fumonisins, coupled with the daily exposure due to tortilla consumption, probably the maximum intake (IDA), would be achieved and may be a factor to be considered for the development of liver cancer in the Mexican population [19].

In the two samples analyzed by UHPC/MS/MS, 22 mycotoxins with an average content of 956.9 μg kg were detected, of which 55.38% corresponded to mycophenolic acid (530.7 μg kg), 20.58% to trichothecenes A and B (Nivalenol, DON, NEO, Fusarenone X, DAS, HT-2, T2, Zearalenone, Zearalenol), 13.77% to fumonisins and the rest to Ochratoxin A with 8.90 μg kg, total aflatoxins 17.0 μg kg, being AFG2 the highest with 8.5 μg kg, Sterigmatocystine with 6.50 μg kg, Roquefortine C with 3 μg kg, Enanthyne 8.65 μg kg, alternarol 17.4 μg kg, methyl alternariol 16.7 μg kg. The micophenolic acid is synthesized by *Penicillum sp*., which can be considered as an emergent mycotoxin, that has recognized immunosuppressive activity [20].

In table 3 it is observed that the mycophenolic acid was the mycotoxin with the highest concentration in both evaluated samples, also the aflatoxins levels between both samples was very similar and those levels are within the national regulation. It is observed that fumonisins levels are within the permitted levels by the European Legislation.

The level of fumonisins and aflatoxins detected in genotype white and yellow maize was variable, in no case outside the national maximum allowable limit for aflatoxins, but it is important to be controlled both mycotoxins due their capacity to affect the biochemical process, in breast cells, even in small amounts and the co-exposure to develop hepatotoxicity; also the unbalance of ceramide has been related by the presence of fumonisins with a greater resistance to insulin, so it can be considered a risk in the development of type 2 Diabetes [21].

4. Conclusions

The presence of *Fusarium verticillioides, Aspergillus flavus, Penicillium sp, Alternaria sp* in maize from Hidalgo, a central state of Mexico, was demonstrated in two agricultural cycles.

The co-contamination of aflatoxins and fumonisins total, had been demonstrated in maize from central states. In addition, the mycophenolic acid as the principal fusariotoxin, present in maize was detected for the first time in the region.

It is necessary to continue the study of the fusariotoxins in maize for human consumption and its interaction with the metabolic syndrome in the Mexican population, since it is suspected that the contamination by fumonisins favors insulin resistance and the development of type 2 Diabetes.

Table 1 Total fumonisin content in 29 samples of white and yellow corn.

Maize genotype	Adaptation zone	Total fumonisins (mg kg)	Variety	Aflatoxins (µg kg)
NB-1	Tropical/subtropical	0.54	white	12
NB-11	Tropical/subtropical	0.54	white	17.7
Eros	Tropical/subtropical	0.23	white	2.1
Py063	Tropical/subtropical	0.23	white	4.0
Zapata	Tropical	0.23	white	3.0
Costeño	Tropical/subtropical	0.23	white	Nd
P4052	Tropical	0.23	white	8.0
Orion	Tropical	0.23	yellow	Negative
NA-35	Tropical	0.23	yellow	Nd
AmCC	Tropical/subtropical	0.40	yellow	Nd
305-49	Tropical/subtropical	0.23	yellow	8.5
H-443	Tropical/subtropical	0.23	yellow	2.1
P-2844	Tropical/subtropical	1.70	yellow	15.2
Tundra	Tropical/subtropical	0.35	yellow	Nd
H382	Tropical	0.23	yellow	15.0
P4032	Tropical	1.0	yellow	Nd
H-515	Tropical	0.23	white	4.5
H-516	Tropical	0.79	white	1.8
30A60	Tropical	0.23	white	14.5
P3055	Tropical/subtropical	0.23	white	5.0
V5335	Tropical/subtropical	1.20	white	6.33
H-377	Tropical/subtropical	0.23	white	2.5
H-374-c	Tropical/subtropical	0.23	white	3.5
ARES	Tropical/subtropical	0.23	white	Negative
San Andrés	Tropical/subtropical	0.43	white	Negative
P-4082	Tropical/subtropical	0.23	white	5.0
30V46	Tropical/subtropical	0.37	white	2.5
Oso	Tropical/subtropical	Nd	white	13
Leopardo	Tropical/subtropical	Nd	white	13

5. Statistic analysis

Table 2 Total fumonisin content in white and yellow native maize.

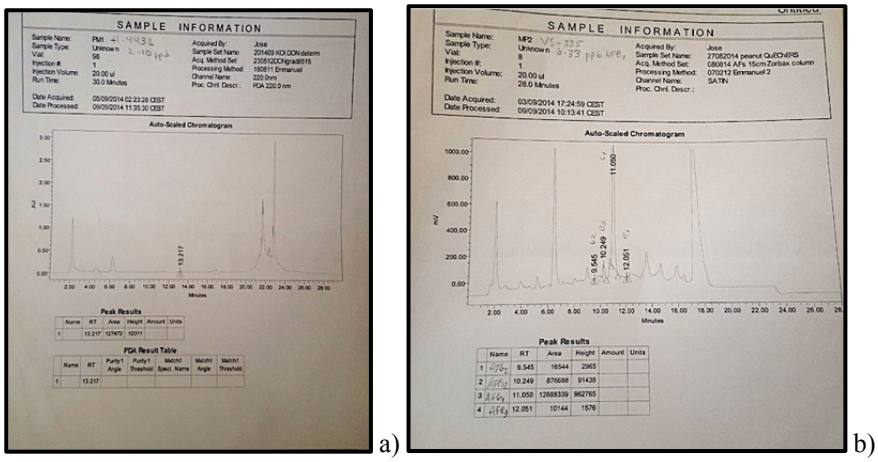
Maize variety	Total Aflatoxins		Total fumonisins Ppm	
	$\bar{x}$	d.e	$\bar{x}$	d.e
Yellow (n=9)	8.16	7.06	0.51	0.51
White (n=20)	6.23	5.26	0.37	0.26
P			<0.01	

$\bar{x}$ = Mean. s.d.= Mean Standard deviation, P=probabilty, 0.05. ppm=parts per million

Table 3 Multimycotoxins analysis.

Sample	Fumonisin (µg/kg)	Ochratoxin A (µg/kg)	Aflatoxin (µg/kg)	Trichothecenes (µg/kg)	Mycophenolic acid (µg/kg)
1	139.30	8.90	16.4	242.90	581.8
2	132.97	8.90	17.7	271	479.7

Fig. 1 Immunochromatographic strip.



a) b)

Fig. 2 Auto-Scaled HPLC Chromatograms.

References

[1] Calderón V., Peña B.S.D., Carvajal M.M. 2007. Cinética de distribución fotoquímica de fumonisina B1 y B2 en maíz mejorado. Tesis Doctoral. CBS. UAM-X.

[2] Peña B.S.D. 2015. Transgene detection (35S y NOS), physical quality, tannins and mycotoxins in maize by human consumption in Mexico. In: Trends in the development and application of educational research in Mexico. Editorial Center for studies and research for teacher development Cenid, 168-190. Guadalajara México. Primera Edición. ISBN: 978-607-8435-02-9.

[3] Del Río-N.B., Velázquez M.O., Sánchez C.C.P., Lara E.A., Berber A., Fanghänel G., Violante R., Tapia C.R., James,W.P., The Encuesta Nacional de Salud (ENSA) 2000 Working Group. 2004. The high prevalence of overweight and obesity in Mexican children. Obesity Research, 12(2): 215-223.

[4] De la Torre Ma. E., Sánchez R. D. 2014. Fumonisinas: síntesis y función en la interacción Fusarium verticillioides- maíz. TIP vol 17, no 1.

[5] Ferrigo D., Raiola A., Caussin R. 2016. Fusarium toxins in Cereals; Occurrence, legislation, factors promoting the appearance and their management. Molecules, 21(627): 1-35.
[6] Norma Oficial Mexicana NOM-187-SSA1/SCFI-2002, Productos y servicios. Masa, tortillas, tostadas y harinas preparadas para su elaboración y establecimientos donde se procesan. Especificaciones sanitarias. Información comercial. Métodos de prueba
[7] Dvorak N.J., Riley R.T., Harris M., McGregor J.A. 2008. Fumonisin mycotoxin contamination of corn-based foods consumed by potentially pregnant women in southern California. Journal of Reproductive Medicine, 53(9): 672-676
[8] International Agency for Research on Cancer (IARC), 2002. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene, volume 82. Working group on the evaluation of carcinogenic risk to humans. IARC Press, Lyon, France, pp. 301-366.
[9] Li F.Q., Yoshizawa T., Kawamura O., Luo X.Y., Li Y.W. 2001. Aflatoxins and fumonisins in corn from the high-incidence area for human hepatocellular carcinoma in Guangxi, China. Journal of Agricultural and Food Chemistry, 49(8): 4122-4126.
[10] Li B., Jiang G., Zhang G., Xue Q., Zhang H., Wang C., Zhao T. 2014. Intake of vegetables and fruit and risk of esophageal adenocarcinoma: a meta-analysis of observational studies. European Journal of Nutrition, 53(7): 1511-1521.
[11] Marasas W.F., Kellerman T.S., Gelderblom W.C., Coetzer J.A., Thiel P.G., Van der Lugt J.J. 1988. Leukoencephalomalacia in a horse induced by fumonisin B1 isolated from Fusarium moniliforme. Onderstepoort Journal of Veterinary Research, 55(4): 197-203
[12] Sun G., Wang S., Hu X., Su J., Huang T., Yu J., Tang L., Gao W., Wang J.S. 2007. Fumonisin B1 contamination of home-grown corn in high-risk areas for esophageal and liver cancer in China. Food Additives and Contaminants, 24(2): 181-185
[13] International Agency for Research on cancer (IARC), 1993. Toxins derived from Fusarium moniliforme: fumonisins B1 and B2 and Fusarin C. In: IARC Monographs on the Evaluation of Carcinogenic Risk to Humans. Lyon: World Health Organization, 56: 445-466.
[14] Códex Alimentarius Commission. Code of practice for the prevention and reduction of mycotoxin contamination in cereals, including annexes on ochratoxin A, zearalenone, fumonisins and tricothecenes (CAC/RCP 51-2003). In Prevention and Reduction of Food and Feed Contamination; FAO, WHO: Rome, Italy, 2003; pp. 1–13.
[15] Peña B. S 2006. Detection of fumonisins in maize (Zea mays L.) by three analytical techniques (HPLC, TLC and ELISA). In Mycotoxins and Phycotoxins Advances in determination, toxicology and exposure management. Eds. H Njapau, Trujillo Sócrates, Hans van Egmond and Park D. Wageningen Academic Publishers, The Netherlands
[16] De Saeger, S. (2011). Determing mycotoxigenic fungi in food and feed. En Determining mycotoxins and mycotoxigenic fungi in food and feed (pp.279-293). Cambridge, UK: Woodhead Publising Limited
[17] Wong R, Harley Y. 2009. Lateral Flow Immunoassay. Springer, Humana Press, New York, NY, USA
[18] Missmer S.A., Suarez L., Felkner M., Wang E., Merrill A.H.J., Rothman K.J., Hendricks K.A. 2006. Exposure to fumonisins and the occurrence of neural tube defects along the Texas-Mexico border. Environmental Health Perspectives, 114(2): 237-241.
[19] European Commission. Commission Regulation (EC) No. 1126/2007 on maximum levels for certain contaminants in foodstuffs as regards Fusarium toxins in maize and maize products. Off. J. Eur. Union 2007, 255, 14–17
[20] Malekinejad H, Aghazadeh A, Rezabakhsh A. (2015). Neurotoxicity of mycotoxins produced in vitro by Penicillium roquefortiisolated from maize and grass silage. Hum.Exp.Toxicol. 34(10), 997-1005.
[21] Babenko, N.A. & Kharchenko, V.S. Biochemistry Moscow (2015) Effects of inhibitors of key enzymes of sphingolipid metabolism on insulin-induced glucose uptake and glycogen synthesis in liver cells of old rats. 80: 104. doi:10.1134/S0006297915010125.

Control of food-borne pathogens growth using bacteriophage

P. Şanlıbaba[1,*] and **B. Uymaz Tezel**[2]

[1] Department of Food Engineering, Engineering Faculty, Ankara University, Gölbaşı, Ankara, Turkey
[2] Food Technology Program, Bayramiç Vocational School, Çanakkale Onsekiz Mart University, Çanakkale, Turkey

Bacteriophages (also called 'phage') were first time isolated in early 1900's. Until late 1930s, they were used to treat infectious diseases in different parts of the world. Improper use of phages and understandable formulations of phage particles reduced the use of phage therapy association with discovering the antibiotics. In recent years, antibiotic resistance is widespread in the world. Also, an increasing resistance to multiple antibiotics has been noted. Phage therapy has again taken into consideration due to the alarming spread of antibiotic resistance. The phage treatment is a new and effective hurdle to control food-borne pathogens including *Listeria monocytogenes, Campylobacter jejuni, Escherichia coli* O157: H7, *Staphylococcus aureus*, and *Salmonella* spp. Food-borne diseases are among the most serious and costly public health concerns worldwide. In despite of good manufacturing, quality control, and hygiene-safety concepts, food-borne illnesses still increased over the past decade. Among food preservation technologies such as physical treatments (heat, pressure, UV, and pulsed light) or chemical sanitizers, phage application has been gained particular interest. One advantage of the phages is that bacterial populations can be controlled selectively in the complex food systems. Phages can be very host specific and also are common in the environment and present in many foods. Phage treatments on food to control of pathogens are to extend shelf-life, to enhance hygienic quality, minimizing the impact on the nutritional and organoleptic properties of perishable food products as they are natural, non-toxic and specific to the bacterial species they infect. Many studies have described the phage biocontrol of these pathogens in a range of foods. This review is a summary of the phage-based strategies for control of food-borne pathogens.

Keywords: biocontrol; food-borne pathogens; phage

1. Introduction

Bacteriophages (also called 'phage') are viruses that specifically infect bacteria. They can be found in every environment where their bacterial hosts are present. They were first discovered independently by Frederick Twort in 1915 and by Felix d'Herelle in 1917 [1, 2, 3]. The global phage population is estimated to be more than 10^{30} [4]. A phage can only attach to a bacterial cell with a specific cell surface receptor. Once attaching to a susceptible cell, phage may undergo one of the two different life cycles (virulent or temperent) [5]. While virulent or lytic phage will enter the cell and then hundreds of copies of the original phage are released, temperate or lysogenic phages do not lyse the host cell and their DNA integrates into the genome of the bacterial cell. Temperate phages can undergo lytic cycles and pick up bacterial host genes including virulence genes and antibiotic resistance genes, so they transfer them to a new bacterial host. For this reason, temperate phages are not selected for phage therapy. Due to their remarkable antibacterial activity, virulent phages have been used as biological tools in humans, animals and plants, particularly against multidrug-resistant bacteria [6, 7].

The food industry has interest in finding alternative approaches to inactivate bacterial pathogens. The use of phage to control pathogens in foods has emerged as a promising tool for food safety in all stages of the food production chain (from farm to folk). Phages may be suitable: i) to prevent or reduce colonization and diseases in livestock (phage therapy), ii) to decontaminate carcasses and other raw products, iii) to disinfect equipment and contact surfaces (phage biosanitation and biocontrol), iv) to extend the shelf life of perishable manufactured foods as natural preservatives (biopreservation), and v) to improve strains used for production, and strain typing. Phages should also be considered in hurdle technology in combination with different preservation methods [8]. In comparison to biocontrol, application of phages for improvement and selection of industrial strains has been poorly described [9].

Phage therapy was first developed early in the last century and showed much promise. One of the most important problems in the phage therapy is the emergence of resistant bacterial strains. These bacteria have always been perceived as a potential obstacle. Most phage resistant cells exhibit changes in their membrane components responsible for specific phage binding. Alteration or deletion of phage receptors from the cell surface protects bacteria from phage attack [10]. In addition, phage preparation can readily be modified in response to changes in bacterial pathogen populations [11]. Development of bacterial resistance and the modification of phage preparations can be managed by using phage cocktails with different combinations of phages that target different receptors on the host bacteria [12].

Phages have been isolated from a wide range of foods like ground beef, pork, chicken and other meat products, chilled and frozen crabmeat, fermented dairy products like cheese and yogurt, and from lettuce and mushrooms. Briefly, phages can be considered a part of the natural microflora of foods [13].

In this review, we aimed to discuss phage application and summarize the current literature on phage based biocontrol to reduce food-borne pathogens in food. In addition, we focused on reducing *Listeria monocytogenes,*

Campylobacter jejuni, Escherichia coli O157:H7, *Salmonella* spp. and *Staphylococcus aureus* on food via phage application.

2. Application of phage in food system

The ideal phages for use as biocontrol agents on pathogenic bacteria must meet some criteria before being considered as suitable candidates. These are: i) their ability to infect specific bacterial target cells and their ability to infect eukaryotic cells, ii) phages generally do not cross bacterial species or genus barrier, and therefore do not affect desirable microorganisms commonly present in foods, gastrointestinal tract or the normal bacterial microbiota, iii) Producing progeny phage without the capacity to integrate into the bacterial genome or transduce bacterial genes from one cell to another, iv) not including virulence genes and antibiotic resistance genes, v) having a broad host range, vi) determined the complete genome sequence of phages, vi) phages being stable over storage and application, vii) phages being amenable to scale up for commercial production, viii) oral feeding studies show no adverse effect, ix) the generally recognized as safe (GRAS) approval for use in foods [13, 14, 15, 16]. Phages should also possess physical characteristics. The stability of phages in foods is important because they may need to be stable under physiochemical conditions of the food to which they are applied. These conditions should be food pH, ion content and osmolarity, thermo tolerance, visible and UV light, osmotic shock, pressure, and processing environments [17].

Inoculum size, timing, phage host range, phage adsorption rate, burst size and density of target bacteria are key elements in the success of phage therapy against food-borne pathogens. There are two ways of bacterial reduction of phages. Passive reduction refers to the reduction of bacteria by the initial phage dose. Therefore, it implies a high number of applied viruses per bacterial cell. In contrast, active reduction can take place with a lower initial dose when phages reach sufficient numbers for bacterial reduction by replication. There are some challenges of phage application on pathogen bacteria. Firstly, a threshold density of bacteria is necessary. It is hard to estimate the time when threshold levels are met. Another challenge is that phages are highly specific for the certain host. Very few phages are able to infect different species, and the host range of most of them includes just a number of strains of one bacterial species [12, 18, 19].

Most phage application studies on food have focused on main emergent food-borne pathogens, such as *L. monocytogenes, C. jejuni, E. coli* O157:H7, *S. aureus*, and *Salmonella* spp. in meats, fruits, vegetables, dairy products and ready-to-eat foods. Contaminating bacteria can get access to food during slaughtering, milking, fermentation, processing, storage or packaging [20]. Bacterial reduction from 0.9 to 6.8 $\log_{10}$ CFU or even their complete elimination in many studies was reported [1]. Phage treatment may also help prevent an incidence of food-borne diseases, reducing food processing (e.g. temperature application) and use of chemical additives (e.g. sulphite and nitrate) [8].

Phage biocontrol has been shown to be more effective in liquid foods than in solid foods [8, 21]. In liquids, even a very small initial number of phages can cause completely lysis of the bacteria in a relatively short time [22]. On the other hand, on solid foods diffusion is limited. Also a greater concentration of phages may be necessary to achieve the same result as in liquid foods. As a conclusion, the concentration of the phages must be sufficiently high to enable contact and subsequent infection, even when bacteria are present at very low numbers. However, there are also some other problems to inhibit food-borne pathogens via phages. The most relevant is the food-matrix [22, 23]. This is a decisive parameter which physically limits the distribution of phage particles in order to reach all targeted bacteria. Moreover, targeted bacteria may be embedded within the rather complex food matrix, thereby shielding them from phage particles. On these grounds, a greater biocontrol effect may be achieved by modifying phage application [21, 23]. It has been suggested that phages can be added by dipping or spraying or as a liquid to a large volume of food. These methods may not be ideal, as they could be wasteful and lead to the potential inactivation of the phage particles. Moreover, when phages are added directly to a batch of food, two major problems may be occurred: 1) the dilution of phages, and 2) the evaluation of bacterial resistance. To overcome of these problems, the addiction of large numbers and volumes of phages using phage cocktails and the regular disinfection of the equipment using effective protocols might help. In addition, immobilized phage may also help to solve these problems [24].

Although phages represent a novel approach, there are no reports of their industrial use to improve safety, even if this "new, ecological and safety" technology may be cheaper than older technologies, since phages can be isolated from the environment and are self-replicating entities [23]. There are several phage preparations commercialized and marketed so far, such as ListShield™ LMP102 (Intralytix, USA), EcoShields™ (Intralytix, USA), SalmoFresh™ (Intralytix, USA), and Listex™ P100 (Micreos Food Safety, The Netherlands) [22]. The first formal approval of a phage–based preparation developed for food safety came during August 2006, when The Food and Drug Administration (FDA) cleared ListShield™ LMP102 for use an antimicrobial agent against *L. monocytogenes* contamination of ready to eat foods [25]. ListShield™ LMP-102 containing a cocktail of six phages is also used for surfaces in food production facilities [26]. Shortly after that approval, during October 2006, a single phage containing preparation (designated Listex™ P100) was approved by FDA for another *L. monocytogenes*-specific phage [4, 25, 27, 28]. The FDA issued a 'no abjection' letter for GRAS designation for both of two commercial anti-listerial phages. Also afterwards, in February 2013, SalmoFresh™ was also approved by as GRAS to control *Salmonella enterica* by the FDA and US

Department of Agriculture (USDA) [28]. Finally, EcoShield™ (ECP-100) is a FDA-cleared commercial phage cocktail of three bacteriophages. It is used to eliminate or reduce food contamination of *E. coli* O157:H7 [3].

2.1 Phage to control *Esherichia coli* O157:H7 contamination

E. coli serogroup O157:H7 known to cause bloody diarrhea and hemolytic uremic syndrome in humans was identified in 1983 [10, 29]. It is a highly virulent food-borne pathogen with an infective dose of ~ 100 cells in humans [30]. Outbreaks have been attributed to food, water, and person-to-person and direct fecal contact. They can be isolated from many foods of animal origin [21]. More than 60 phages specific *E. coli* O157:H7 have been reported. The commercial EcoShield™ is based on ECP-100 and contains a phage concentration of at least 10^{11} PFU/mL [31]. This phage preparation was approval by FDA as commercial phage cocktail of three bacteriophages to eliminate or reduce food contamination of *E. coli* O157:H7 [3].

Phages DT1 and DT6, either alone or mixed in a cocktail, were evaluated for their efficiency to inhibit the growth of *E. coli* strains during milk fermentation by Tomat et al. [16]. In absence of phages, bacterial strain reached 4-6 $\log_{10}$ CFU/mL at 5-6 h. *E. coli* DH5α and O157:H7 STEC strains were rapidly and completely inactivated by phage DT1, while 0157:H7 STEC was completely inactivated either by DT1 or by DT6 after 8 h. By the way, pH values evolved falling to 4.5 at 8 h and 4.0 at the end of the fermentation process. All phage titres excluding the phage cocktail constant or increased slightly throughout the first 8 h, with a subsequent decrease between 8 and 24 h. The authors have said that the low pH and accumulated lactic acid might be related the partial phage inactivation.

Mclean et al. [21] also studied that three phages were investigated for their ability to inhibit the growth of three strains of *E. coli* in ultra-high-temperature treated and raw bovine milk. A cocktail of the three phages completely inhibited *E. coli* ATCC 25922, *E. coli* O5: H- and *E. coli* O127:H6 in UHT milk at 25 °C. Similar results were obtained from raw milk. No re-growth of bacterial strains was observed in any of the phage treated milk samples. Under refrigeration temperatures, the phage cocktails inhibited all three strains of *E. coli* to below the level of detection within 24 h of incubation. Furthermore, phage titers did not fluctuate by more than 1 $\log_{10}$ PFU/mL. The phage cocktail containing two phages eliminated ATCC 25922 and O127:H6 in raw milk within 3 h and 6 h of incubation, respectively, at 25 °C and in refrigerated samples. Furthermore, phage titers did not fluctuate by more than 0.5 $\log_{10}$ PFU/mL. In contrast, the phage cocktails completely inhibited O5: H- in UHT milk at both temperatures. In raw milk, this phage cocktails initially inhibited growth of O5: H- but re-growth occurred for 9 h at 25 °C. The researchers have demonstrated that differences in milk composition (proteins or oil globules), microbiota, and lower target cell and phage concentrations are effective on phages against O5: H-.

Tomat et al. [23] evaluated that the reduction of viable cells of *E. coli* O157:H7, and enteropathogenic *E. coli* strains on meat after exposure to phage applications at 5 °C and 24 °C for 3, 6, and 24 h. Two phages (DT1 and DT6) were selected. When enteropathogenic *E. coli* and O157:H7 strains were tested, viable cell reduction of 0.67 $\log_{10}$ and 0.77 $\log_{10}$ after 3 h incubation and 0.80 $\log_{10}$ and 1.15 $\log_{10}$ after 6 h. They implied that higher reductions were observed at higher temperature, probably due to the active growth of bacteria allowing an efficient phage replication. In addition, the phage cocktail at a higher number of different phages was able to of further reduce viable cell counts of O157:H7 than individual phages on meat products.

The ECP-100 was examined for its ability to reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by Abuladze et al. [27]. Treatments (5 min) of the contaminated hard matrices with the ECP-100 preparation containing different concentrations of phages (10^{10}, 10^{9}, 10^{8} PFU/mL) resulted in significant reduction of 99.99%, 98%, and 94%, respectively. Samples of broccoli, tomato, and spinach, and also ground beef samples were contaminated with 710, 650, 14000 and 3400 CFU of O157:H7/g, respectively. The observed reduction ranged from 94% (at 120 h post-treatment tomato samples) to 100% (at 24 h post-treatment of spinach samples). The authors found that naturally occurring phages may be useful for reducing contamination of various food samples by O157:H7.

O'Flynn et al. [29] were identified that two distinct lytic phages and a cocktail of three phages were evaluated to lyse the E. coli O157:H7. Phages resulted in a 5 $\log_{10}$ unit reduction of pathogen numbers in 1 h at 37 °C. In an initial meat trial experiment, the phage cocktail completely eliminated O157:H7 from the beef surface. Similarly, single phage application caused a 3 $\log_{10}$ unit reduction in the number of viable cells within 2 h at 37 °C. When incubated at 37 °C for all phages when they applied individually or as a cocktail, the culture started to grow again within 2 to 3 h. But, this re-growth was not observed for incubation at 30 °C.

Sharma et al. [32] determined that the efficacy a mixture of three O157:H7 specific phages (ECP-100) in reducing the number of viable *E. coli* O157:H7 on contaminated fresh cut iceberg lettuce and cantaloupe. Bacterial strain was spot inoculated on lettuce pieces with a population of 3.76 $\log_{10}$ CFU/ cm^2, allowed to dry, and then sprayed ECP-100 to deliver 7.98 $\log_{10}$ PFU/cm^2 to lettuce stored for 2 days at 4 °C. Spraying reduced the number of viable of O157:H7 on fresh cut lettuce. Furthermore, bactericidal effect occurred very quickly after spraying. Cut pieces of cantaloupe were also spot inoculated with O157:H7 (4.55 $\log_{10}$ CFU/mL) and treated with ECP-100 (6.69 $\log_{10}$ PFU/mL) and then stored at 4 °C or 20 °C for up to 7 days. Populations of O157:H7 on lettuce treated with ECP-100 on 0, 1, and 2 days (0.72, <0.22, and 0.58 $\log_{10}$ CFU/cm^2 of lettuce) were significantly lower than those treated with the control (2.64, 1.79, and 2.22 $\log_{10}$ CFU/cm^2), respectively. Populations on cut cantaloupes treated with ECP-100 on days 2, 5, and 7 (0.77,

1.28, and 0.96 $\log_{10}$ CFU/mL) and then stored at 4 °C were significantly lower than those treated with the control (3.34, 3.23, and 4.09 $\log_{10}$ CFU/mL), respectively.

2.2 Phage to control *Salmonella* contamination

Salmonella is a gram negative, rod-shaped bacterium. Salmonellosis is one of the most commonly reported zoonotic diseases in many countries [13]. Animal-derived food, including eggs, egg products and raw or undercooked meats are the principal source of infection. Vegetables products are also been reported [1]. Although more than 2500 serovars of *Salmonella* enterica have been identified, the human infections are caused by a limited number of serovars *S.* enteritidis, *S.* newport and *S.* typhimurium belong to the most common serovars [33, 34]. According to the most recent report, approximately 1.4 million infections per year are estimated, resulting in 400-600 deaths. Therefore, the development and evaluation of new strategies for the control of *Salmonella* are urgently needed [13, 35]. Apart from the traditional use of heat and chemicals, many emerging technologies, phages were reported to be successful against *Salmonella* [33, 35]. Among the phages to inhibit *Salmonella enterica*, SalmoFresh™ and SalmoLyse™ were approved by as GRAS by the FDA and US Department of Agriculture (USDA) [28]. SalmoFresh™ is prepared with a cocktail of naturally occurring lytic phages and inhibited *Salmonella* including strains belonging to the most common pathogenic serotypes: Typhimurium, Enteriditis, Heidelberg, Newport, Hadar, Kentuck and Thomson. SalmoLyse™ is a reformulated phage cocktail derived from SalmoFresh™ in which two of six phages in the original cocktail have been replaced [31]. Most of the many studies have focused on the use of phage to reduce carriage of *Salmonella* in poultry rather than as a food additive [36].

Galarce et al. [1] evaluated the effectiveness of five phages applied as a cocktail to reduce the counts of *S. enterica* serotype enteritidis (SE) in the two types of processed meat products. Each sample was contaminated with SE, treated with a phage cocktail and then incubated for ten days at 18 °C and 4 °C. A significant reduction in bacteria was obtained on days 3, 6 and 10 incubated at 18 °C (from 0.48 to 2.12 $\log_{10}$ CFU/g) and at 4 °C (from 0.23 to 2.06 $\log_{10}$ CFU/g). They implied that the bacterial reduction obtained in their study was lower using the same phage cocktail in fresh chicken and turkey breast. Industrial processing of the food, changes the chemical composition, moisture, water activity and pH, bacterial growth and phage activity are responsible for these differences.

Guenther et al. [13] evaluated the reduction of *S.* Typhimurium in different ready-to eat-foods by virulent phage FO1-E2. Samples were inoculated with 10^3 CFU/g *Salmonella* cells and treated with 10^8 PFU/g phage and incubated for 6 days at 8 °C or 15 °C. At 8 °C, no viable cells remained following phage application, corresponding to a more than 3 $\log_{10}$ unit reduction. At 15 °C, application of phage lowered *S.* Typhimurium counts by 5 $\log_{10}$ units on turkey deli meat and in chocolate milk and by 3 $\log_{10}$ units on hot dogs and in seafood. They observed that phage resistant *Salmonella* strains appeared at the end of the incubation period. They stressed the environmental condition with an incubation temperature of 15 °C.

Hungaro et al. [34] studied that five phages with inoculating in chicken skin used to reduce *S.* enteritidis at 37 °C and 25 °C. The chicken skin sections exhibited natural contamination of approximately 10^5 CFU/cm^2 of *S.* enteritidis. All five phages were able to reduce *S.* enteritidis growth at 10^9 PFU/mL in both temperatures evaluated. The phage cocktail at 10^9 PFU/mL applied to chicken skin for 30 min achieved significantly reduction in *S.* enteritidis counts. When *S.* enteritidis was challenged with phages at 10^3 PFU/mL and 10^6 PFU/mL, no reduction in bacterial growth were observed. They demonstrated that bacterial growth inhibition was clearly phage concentration-dependent.

Four phage treatments were used against *Salmonella* from poultry carcass rinse samples by Higgins et al. [37]. In the first two experiments, the highest concentration of phage (10^{10} PFU/mL) applied significantly reduced *S.* enteritidis recovery between 50 and 100% as compared with the control rinse water sample. In the experiments 3 and 4, broiler carcasses were inoculated with 31CFU/g *S.* enteritidis, sprayed with selected concentrations of phage. Similarly results were found that application of 10^8 or 10^{10} PFU/mL phage reduced the frequency of pathogen counts. They suggest that the treatment with large number of phage is desirable.

The effectiveness of a phage cocktail was determined in four different food matrices (pig skin, chicken breast, fresh eggs and packaged lettuce) contaminated with *S.* typhimurium and *S.*enteritidis by Spricigo et al. [38]. A significant bacterial reduction (>4 and 2 $\log_{10}$/cm^2) was obtained in pig skin sprayed with the phage cocktail and then incubated at 33 °C for 3 and 6 h. These times were chosen because both of them are the duration of the pre-slaughter withdrawal period. Chicken breast were dipped for 5 min in a solution containing the phage cocktail and then maintained for 7 days at 4 °C. By day 7 of treatment, *S.* typhimurium concentration was 2.2 $\log_{10}$ CFU/g lower than the initial concentration whereas the reduction in *S.* enteritidis counts was greatest on day 5 post-treatment. Only a minor reduction of the bacterial concentration (0.9 $\log_{10}$ CFU/cm^2 in the concentration of both *Salmonella* serovars) was achieved in fresh eggs sprayed with the phage cocktail and then incubated at 25 °C for 2 h. Likewise, there was a reduction of the *Salmonella* concentration in lettuce. The cocktail was more effective in *S.* typhimurium, with a decrease of 3.4 and 3.9 $\log_{10}$ CFU/g after 30 and 60 min than in *S.* enteritidis, in which a significant reduction of 1.9 and 2.2 $\log_{10}$ CFU/g after 30 and 60 min of treatment respectively was observed.

Wong et al. [39] showed that inoculation of 10^{12} PFU/mL of the phage in the chickens challenged with 10^{10} CFU/mL of *S.* typhimurium was able to reduce the *S.* typhimurium. The *Salmonella* count reduced to 2.9 log10 CFU/mL within 6 h of post-challenge. Zinno et al., (2014) focused on the use of phage P22 to eradicate *S.* typhimurium

in different foods. Bacterial growth was monitored for 24 and 48 h at 4 °C. The cell loads at the initial time of in different treated foods did not show significant differences. On the contrary, significant difference in the mean value of viable count at 24 and 48 h was shown. When 10^4 CFU/g host inoculums were used, 2 - 3 $\log_{10}$ bacterial inactivations were detected in all food matrices after 48 h. Their results also showed that in liquid foods there was a significant reduction of the bacterial population after 48 h, while in other foods the cell loads were not affected. They emphasized that phage application was not enough to inactivate the entire population of pathogen bacteria. So, combination of phage with other natural antimicrobials such as bacteriocins or essential oils should be encouraged. In addition, their results indicated that phage ability against the pathogen bacteria is more effective in liquid foods than the solid.

2.3 Phage to control *Listeria monocytogenes* contamination

Listeria is ubiquitously found in the environment, and transmission usually occurs via contaminated food and water [22, 31]. Among the *Listeria* species, *L. monocytogenes* are the greatest concern, owing to grow at low temperatures, survive in high salt environments and produce biofilms. Because of these properties, it is a high risk organism for food production [40]. Listeriosis is associated with mortality rates up 30%. To date, more than 500 *Listeria* phages have been isolated and characterized. Phage Listex™ P100 has received GRAS status by FDA/USDA for use in food materials. P100 was isolated from a sewage effluent sample of a dairy processing plant in Germany [41]. In addition, the phage preparation LMP-102 has been commercialized as ListShield™ LMP-102 containing a cocktail of six phages. It has been shown to be effective against 170 different strains of *L. monocytogenes,* reducing significantly from 10 to 1000 fold [31]. It was also approved FAD and USDA for application on food and surfaces in food production facilities as microbial pesticide [42].

Soni et al. [4] studied that the influence of phage dose, phage contact time, and storage temperature on the listericidal activity of phage P100 in reducing *L. monocytogenes* loads on the surface of fresh channel catfish fillet. The fresh catfish fillet samples inoculated with ~ 4.3 $\log_{10}$ CFU/g of a two serotype mix of *L. monocytogenes* cells and then surface treated with phage P100. The effect of phage P100 concentrations of 10^3, 10^5, and 10^7 PFU/g against *L. monocytogenes* on catfish fillet pieces within 2 h at room temperature. P100 treatment of 10^7 PFU/g resulted in an average of 1.6 $\log_{10}$ CFU/g reduction in *L. monocytogenes* counts. At density of 10^5 PFU/g of phage, there was a slight reduction of 0.4 $\log_{10}$ CFU/g reduction in *L. monocytogenes* loads. But, there was no reduction in *L. monocytogenes* counts at 10^3 PFU/g of phage P100 dose. The reduction in *L. monocytogenes* counts with the phage P100 dose of $2x10^7$ PFU/g (7.3 $\log_{10}$ PFU/g) was 1.4-2.0 $\log_{10}$ CFU/g at 4 °C, 1.7-2.1 $\log_{10}$ CFU/g at 10 °C, and 1.6-2.3 $\log_{10}$ CFU/g at 22 °C on raw catfish fillet. The phage contact time of 30 min was adequate to yield greater than 1 $\log_{10}$ CFU/g reduction in *L. monocytogenes*, whereas 15 min contact time with phage yielded less than 1 $\log_{10}$ CFU/g reduction of *L. monocytogenes* loads on catfish fillet. The authors suggested that P100 titer was stable on catfish fillet samples, and the cell counts were still maintained over 10 day shelf life at 4 °C or 10 °C by P100 treatment.

Oliveira et al. [22] investigated that the efficacy of phage P100 to control *L. monocytogenes* growth on melon, pear, and apple products (juices and slices) stored at 10 °C. Phage treatment was effective on melon followed by pear, but no effect on apple products was observed. Reduction of about 1.50 and 1.00 $\log_{10}$ CFU/plug for melon and pear slices were found. In juices, higher reductions were obtained in melon (8.00 $\log_{10}$ CFU/mL) followed by pear (2.10 $\log_{10}$/mL) after 8 days of storage. The bacterial cells in apple juice was unaffected by phage treatments. Consequently, phage treatment decreases *L. monocytogenes* growth on fruit slices and juices. However, these reductions were greater in fruit juices than fruit slices. The authors said that the pH differences may be a major factor contributing to the differences in the bacterial populations on the fruit slices and juices. In addition, phages are seemingly immobilized after addition to solid surface. Therefore, they could not come into contact with the surviving bacteria through limited diffusion.

Bigot et al. [43] were aimed to control of *L. monocytogenes* on ready to eat foods. First of all, broth assays showed that A511 phages added at $5.2x10^7$ PFU/mL prevented the growth of *L. monocytogenes* (10^8 CFU/mL) at 30 ° for 7 h. In the presence of phages, there was a reduction of cell concentration after four to five hours of incubation, but re-growth occurred after 24 h. At the same temperature, but on the surface of vacuum-packed ready to eat chicken breast roll, there was an immediate 2.5 $\log_{10}$ CFU/cm^2 reduction in pathogen concentration following addition of phages and then re-growth. However, when similar experiments were performed at 5 °C, there was an initial 1.5 $\log_{10}$ CFU/cm^2 reduction in host cell concentration. In addition, re-growth of bacterial cell was prevented over 21 days incubation.

Guenther and Loessner [44] showed that two different soft-ripened cheese models (white mold and red-smear) were established in the laboratory to evaluate the potential of A511 phage for controlling *L. monocytogenes* on the cheese surfaces. The surfaces of cheese were inoculated with 10^1-10^3 CFU/cm^2 bacterial cells. Phage was applied at defined time points at thereafter, in single or repeated treatments, at 10^8 or 10^9 PFU/cm^2. With 10^3 CFU/cm^2 of bacterial cell concentrations and a single dose of A511 (10^8 PFU/cm^2) on white-mold cheese samples, viable counts dropped 2.5 logs at the end of the 21 day ripening period. Repeated phage application did not further inhibit the bacteria. A single higher dose (10^9 PFU/cm^2) was found to be more effective. On red-smear cheese ripened for 22 days, *Listeria* counts were down by more than 3 logs. Repeated application of A511 further delayed re-growth of *Listeria*, but did not affect bacterial counts after 22 days. With a lower initial *Listeria* contamination, viable counts dropped below the limit of detection.

Rossi et al. [45] investigated that *L. monocytogenes* 1/2a was inoculated in Brazilian fresh sausage ($2.1x10^4$ CFU/g) with a phage P100 added thereafter ($3x10^7$ PFU/g). Samples were analyzed immediately and then stored at 4 °C for 10 days. P100 reduced bacterial cell counts by 2.5 $\log_{10}$ units at both 0 and 10 days. In spite of this, the bacterial cells increased over the 10 day storage. This result demonstrated the psychotropic characteristic of the bacteria.

Soni et al. [46] analyzed that phage P100 was evaluated against *L. monocytogenes* cold growth in queso fresco cheese. When 9 $\log_{10}$ CFU/mL of stationary phase cells of *L. monocytogenes* exposed to P100, there was a 3 to 5 $\log_{10}$ CFU/mL reduction after 24 h at 4 °C in vitro study. In cheese samples, the bacterial population increased from the initial 3.5 $\log_{10}$ CFU/cm^2 to 7.7 $\log_{10}$ CFU/cm^2 in 28 days at 4 °C. Treatment with 7.8 $\log_{10}$ PFU/cm^2 of phage P100 showed strong listericidal effect initially by reducing *L. monocytogenes* counts by 2 to 3.5-4 $\log_{10}$ CFU/cm^2.

2.4 Phage to control *Campylobacter jejuni* contamination

Campylobacter was first described in 1880 by Theodore Escheric. These bacteria are curved, S-shaped or spiral gram negative rods, which can only multiply in warm blooded animals such as poultry, pigs, cattle and wild birds. *C. jejuni, C. coli* and *C. lari* are the most frequently reported in *Campylobacter* species [47]. In the developed world, *C. jejuni* is a leading cause of food-borne gastrointestinal disease [48]. Human infections arise from uncooked poultry meat, hand-to-mouth transfer in the kitchen, and cross-contamination of other foods [19]. A significant traceable source of human infection is poultry. Cooking is the key to eliminating the risk of *Campylobacter* enteritis from poultry dishes. To safely cook such dishes, critical core temperature of 68-70 °C must be reached and held for periods as long as 45 min which can result in unacceptable sensory characteristics. The use of organic acid was found to cause a colour change or bleaching of the lever surface [49]. More useful mechanisms to control *Campylobacter* is the use of host-specific phage [50]. *Campylobacter* phages have been isolated from several different sources such as sewage, pig and poultry manure, abattoir effluents, broiler chickens and retail poultry [51]. From the intestinal content of poultry, purified phages may be transferred to the surface of poultry meat, at slaughter. They are able to survive for more than 10 days and naturally present on foods for human consumption. *Campylobacter* specific phages offer the prospect of a sustainable measure to reduce the numbers of *Campylobacter* colonization in poultry [52]. This is promising and results have indicated *Campylobacter* reductions of up to three $\log_{10}$ units have been achieved by phage application [53]. To date, there are more than 170 phages of *Campylobacter* species reported [36]. There are only a few reports on phage biocontrol against *Campylobacter*, with all the studies being conducted on poultry [51].

El-Shibiny et al. [18] also studied the effect of phage CP220 on *C. jejuni* and *C. coli* colonized broiler chickens. A 2 $\log_{10}$ CFU/g decline in cecal *Campylobacter* counts was observed after 48 h in birds colonized with *C. jejuni* HPC5 and administered with a single 7 $\log_{10}$ PFU oral dose of CP220. To achieve a similar reduction in *Campylobacter* reduction in *C. coli* OR12-colonized birds, a 9 $\log_{10}$ PFU dose of CP220 was required.

Kittler et al. [19] used to a phage cocktail of *Campylobacter* reduction on three commercial broiler farms. One day after phage application, *Campylobacter* counts were reduced under detected limit (<50 CFU/g) in fecal samples. At slaughter, a significant reduction of >$\log_{10}$ 3.2 CFU/g cecal content was detected. They also revealed that maximum reduction of *Campylobacter* at the slaughterhouse might be achieved by phage application 1 to 4 days prior to slaughter.

Fischer et al. [47] inoculated commercial broilers with 10^4 CFU/bird of a *C. jejuni* field strain. Then, each group of birds was treated with 10^7 PFU/bird of a single phage or four phage cocktails. Finally, they demonstrated that the *Campylobacter* load was permanently reduced by the phage cocktail as well as by the single page. The reduction was reached a maximum of $\log_{10}$ 2.8 CFU/g cecal contents.

Firlieyanti et al. [49] showed that the application of broad host range phages (8 $\log_{10}$ PFU/g) to liver homogenates containing *C. jejuni* strains of diverse origin at 4 °C resulted in modest but significant reductions in the viable counts ranging from 0.2 to 0.7 $\log_{10}$ CFU/g.

Atterbury et al. [50] observed that the enumeration of campylobacters from chicken ceca in the presence of phage (mean $\log_{10}$ 5.1 CFU/g) was associated with a significant reduction in numbers compared to samples with *Campylobacter* alone (mean $\log_{10}$ 6.9 CFU/g). The detection limit was determined as $\log_{10}$ 2.0 PFU/g *Campylobacter* CFU/g of cecal contents and similarly for phage $\log_{10}$ 2.0 PFU/g of cecal contents.

Carvalho et al. [51] studied the efficacy of a phage cocktail in reducing the levels of colonization by both *C. coli* and *C. jejuni* in broiler birds. They showed that approximately 2 $\log_{10}$ CFU/g reduction of both *Campylobacter* strains was occurred. They also implied a 30 fold reduction in the incidence of campylobacteriosis associated with consumption of chicken meals could be leaded according to mathematical models.

Atterbury et al. [54] determined the efficacy of phages to reduce the number of recoverable *C. jejuni* on artificially contaminated chicken skin. A high concentration of the phage (10^7 PFU) was found more effective in reducing the recoverable *C. jejuni* in frozen chicken. The researchers in the study recommended combining freezing and phage treatment are ensured further falls in *Campylobacter* prevalence on broiler carcasses.

Carrillo et al. [55] determined that CP8 and CP34 were applied at different dosages, to 25-day-old broiler chickens experimentally colonized with the *C. jejuni* broiler isolates. Phage treatment of *C. jejuni* resulted in *Campylobacter* counts falling between 0.5 and 5 $\log_{10}$ CFU/g of cecal contents compared to untreated controls over a 5-day period post administration. The optimum dose for phage therapy was reported to be 7 $\log_{10}$ PFU, with the higher (9 $\log_{10}$ PFU) and lower doses (5 $\log_{10}$ PFU) of phage being generally less effective.

2.5 Phage to control *Staphylococcus aureus* contamination

S. aureus has been responsible for outbreaks associated with milk and dairy products. In addition, particularly these strains can able to produce heat stable enterotoxins [56]. This strain is dependable for the 1-9 % outbreaks [57]. Biocontrol of staphylococci has focused considerably on the prevention and treatment of mastitis and improvement of animal health [40].

Bueno et al. [56] studied that two *S. aureus* lytic phages were evaluated for their potential as biocontrol agents against this pathogenic bacteria in both fresh and hard-type cheeses. Pasteurized milk was contaminated with pathogen (about 10^6 CFU/mL) and a cocktail of the lytic phages (about 10^6 PFU/mL) was also added. In both types of cheeses, the presence of phages resulted in a notorious decrease of S. aureus viable counts during curdling. In fresh cheeses, a reduction of 3.83 $\log_{10}$CFU/g of *S. aureus* in 3h and viable cells were under the detection limits after 6 h. At the end of the curdling process, the pathogen strain was undetected and no re-growth was occurred during the cold storage. In hard cheeses, the presence of phages resulted in a continuous reduction of *S. aureus* counts. In curd, viable counts of *S. aureus* were reduced 4.64 $\log_{10}$ CFU/g. The authors also emphasized that starter strains were not affected by the presence of phages in the cheese making processes and cheese maintained their expected physic-chemical properties.

Garcia et al. [57] determined the ability of specific phages of S. aureus growth in curd manufacturing processes. A cocktail of two lytic phages produced a complete elimination of 3 x 10^6 CFU/mL of the pathogen in ultra high temperature whole milk at 37 °C. Furthermore, the frequency of emergence of phage insensitive mutants was reduced up to 200-fold in the presence of phage cocktail. In acid curd, the pathogen was not detected after 4 h of incubation at 25 °C, whereas pathogen clearance was achieved within 1 h of incubation at 30 °C in renneted curd. The authors indicated that even though the phage cocktail was partially inactivated by low pH, it was able to completely eradicate viable S. aureus cells in curd made of heavily contaminated milk.

Haddad et al. [58] selected two anti S. aureus phage cocktails, each containing three phages. The use of these phages did not trigger over production of S. aureus enterotoxin C. Cocktail 1 was first tested against *S. aureus* on Cheddar cheese and the latter inoculated at 10^6 CFU/mL of milk. At the initial milk maturation and coagulation steps, no significant bacterial reduction was observed. Reduction of 1 and 2 $\log_{10}$ units of the staphylococcal counts were observed from the initial step until the coagulation step. At the coagulation step, the whey removal and the cheddaring step helped increase phage efficiency and dropped the staphylococcal concentration by 1.3 and 4 $\log_{10}$ units per g of curd. After a 14 day ripening, all cheeses with added phages did not containing *S. aureus* cells. For the cocktail 2, a significant decrease in the concentration of pathogen began at maturation. Similarly to the phage cocktail 1, no cells were detected above the limit of detection of 100 CFU/mL in all cheeses with the added phage cocktail 2 after 14 days.

Tabla et al. [59] researched a cocktail of two phages performance against *S. aureus* in milk by high hydrostatic pressure (HHP) treatments. Four hundred MPa was found to be most suitable pressure to be used in combination with these phages. Two different levels of bacterial initial contamination (10^4 and 10^6 CFU/mL) were tested, and a synergistic effect between HHP and phages was observed in both cases. The combine treatment was able to reduce the initial pathogen contamination below the detection limit. The authors particularly emphasized that phages can be regarded as a valuable hurdle on minimally processed food.

3. Conclusion

Recently, there has been an increased interest in application of phage as an alternative antimicrobial chemotherapy in various fields such as human infections, food safety, agriculture and veterinary sciences. The scientific literatures demonstrate that phages have been suggested as an alternative approach to effectively reduce the amount of food-borne pathogens in food safety. It is necessary to determine the criteria for application of phages in the control of pathogen bacteria in food and therefore, all newly isolated phages with the potential application in antibacterial therapy should be characterized in detail.

Latest developments in genome sequencing and bioinformatic technologies have allowed a large number of phage studies to be carried out at the genomic level. These studies are provided further opportunities for applications in novel biocontrol agents and phage therapy. The results of such studies appear to be encouraging. But research is still needed to thoroughly because there are many questions that remain to be answered through the collection of scientific data such as phage-bacteria interactions and ecology, phage efficacy, under different environment conditions of food producing, and phage resistance.

References

[1] Galarce N, Escobar B, Rojas V, Navarro C, Turra G, Robeson J, Borie C. Application of a virulent bacteriophage cocktail leads to reduction of *Salmonella enterica* serovar Enteritidis counts in processed meat products. Biocontrol Science and Technology. 2016; 26: 462-75.

[2] Clokie MRJ, Millard AD, Letarov AV, Heaphy S. Phages in nature. Bacteriophage. 2011; 1: 31-45.

[3] Sabouri S, Sepehrizadeh Z, Amir-Rostami S, Skurnik M. A mini review on the in vitro and in vivo experiments with anti-*Escherichia coli* O157:H7 phages as potential biocontrol and phage therapy agents. International Journal of Food Microbiology. 2017; 243: 52-57.
[4] Soni KA, Nannapaneni R, Hagens S. Reduction of *Listeria monocytogenes* on the surface of fresh channel catfish fillets by bacteriophages Listex P100. Foodborne Pathogens and Disease. 2010; 7; 427-34.
[5] Garcia P, Rodriguez L, Rodriguez A, Martinez B. Food biopreservation: promising strategies using bacteriocins, bacteriophages and endolysins. Trends in Food Science and Technology. 2010; 21: 373-82.
[6] Hooton SPT, Atterbury RJ, Connerton IF. Application of a bacteriophage cocktail to reduce *Salmonella* Typhimurium U288 contamination onpig skin. International Journal of Food Microbiology. 2011; 151: 157-63.
[7] Grant A, Hashem F, Parveen S. *Salmonella* and *Campylobacter*: Antimicrobial resistance and bacteriophage control in poultry. Food Microbiology. 2016; 53: 104-09.
[8] Zinno P, Devirgiliis C, Ercolini D, Ongeng D, Mauriello G. Bacteriophage P22 to challenge *Salmonella* in foods. International Journal of Food Microbiology. 2014; 191: 69-74.
[9] Krasowska A, Biegalska A, Augustyniak D, Los M, Richert M, Lukaszewicz M. Isolation and characterization of phages infecting Bacillus subtilis. Biomed Research International. 2015; Article ID 179597.
[10] Tanji Y, Shimada T, Yoichi M, Miyanaga K, Hori K, Unno H. Toward rational control of *Escherichia coli* O157:H7 by a phage cocktail. Applied Microbiology and Biotechnology. 2004; 64: 270-74.
[11] Viazis S, Akthar M, Feirtag J, Diez-Gonzalez F. Reduction of *Escherichia coli* O157:H7 viability on hard surfaces by treatment with a baceriophage mixture. International Journal of Food Microbiology. 2011; 145: 37-42.
[12] Connerton PL, Timms AR, Connerton IF. *Campylobacter* bacteriophages and bacteriophage therapy. Journal of Applied Microbiology. 2011; 111: 255-65.
[13] Guenther S, Herzig O, Fieseler L, Klumpp J,Loessner MJ. Biocontrol of *Salmonella* Typhimurium in RTE foods with the virulent bacteriophage F01-E2. International Journal of Food Microbiology. 2012; 154: 66-72.
[14] Hagens S, Loessner MJ. Bacteriophage for biocontrol of foodborne pathogens: Calculations and considerations. Current Pharmaceutical Biotechnology. 2011; 11: 58-68.
[15] Bardina C, Spricigo DA, Cortes P, Llagostera M. Significance of the bacteriophage treatment schedule in reducing *Salmonella* colonization of Poultry. Applied and Environmental Microbiology. 2012; 78: 6600-07.
[16] Tomat D, Migliore L, Aquili V, Quiberoni A, Baalague C. Phage biocontrol of enteropathogenic and shiga toxin-producing *Escherichia coli* in meat products. Frontiers in Cellular and Infection Microbiology. 2013: 3; article 20.
[17] Hudson JA, Billington C, Carey-Smith G, Greening G. Bacteriophages as biocontrol agents in food. Journal of Food Protection. 2005; 68: 426-37.
[18] El-Shibiny A, Scott A, Timms A, Metawea Y, Connerton PL, Connerton IF. Application of a group II *Campylobacter* bacteriophage to reduce strains of *Campylobacter jejuni* and *Campylobacter coli* colonizing broiler chickens. Journal of Food Protection. 2009; 72: 733-40.
[19] Kittler S, Fischer S, Abdulmawjood a, Glünder G, Klein G. Effect of Bacteriophage Application on *Campylobacter jejuni* Loads in Commercial Broiler Flocks. Applied and Environmental Microbiology. 2013; 79: 7525-33.
[20] Garcia P, Martinez B, Obeso JM, Rodriguez A. Bacteriophages and their application in food safety. Letters in Applied Microbiology. 2008; 47: 479-85.
[21] Mclean SK, Dunn LA, Palombo EA. Phage Inhibition of *Escherichia coli* in ultrahigh temperature treated and raw milk. Foodborne Pathogens and Disease. 2013: 10; 956-62.
[22] Oliveira M, Vinas I, Colas P, Anguera M, Usall J, Abadias M. Effectiveness of a bacteriophage in reducing *Listeria monocytogenes* on fresh cut fruits and fruit juices. Food Microbiology. 2014; 38: 137-42.
[23] Tomat D, Mercanti D, Balague C, Quiberoni A. Phage biocontrol of enteropathogenic and Shiga toxin-producing *Escherichia coli* during milk fermentation. Letters in Applied Microbiology. 2013: 57; 3-10.
[24] Anany H, Chen W, Pelton R, Griffiths MW. Biocontrol of *Listeria monocytogenes* and *Escherichia coli* O157:H7in meat by using phages immobilized on modified cellulose membranes. Applied and Environmental Microbiology. 2011; 77: 6379-87.
[25] Sulakvelidze A. Using lytic bacteriophages to eliminate or significantly reduce contamination of food by foodborne bacterial pathogens. Journal of the Science of Food and Agriculture. 2013; 93: 3137-46.
[26] Soni KA, Nannapaneni R. Bacteriophage significantly reduces *Listeria monocytogenes* on raw salmon fillet tissue. Journal of Food Protection. 2010; 73; 32-38.
[27] Abuladze T, Li M, Menetrez MY, Dean T, Senecal A, Sulakvelidze A. Bacteriophages reduced experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157:H7. Applied and Environmental Microbiology. 2008; 74: 6230-38.
[28] Oh H, Seo DJ, Jeon SB, Park H, Jeong S, Chun HS, Oh M, Choi C. Isolation and characterization of *Bacillus cereus* bacteriophages from foods and soil. Food and Environmental Virology. 2017; 9.
[29] O'Flynn G, Ross RP, Fitzgerald GF, Coffey A. Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7. Applied and Environmental Microbiology. 2004; 70: 3417-24.
[30] Raya RR, Varey P, Oot RA, Dyen MR, Callaway TR, Edrington TS, Kutter EM, Brabban AD. Isolation and characterization of a new T-even bacteriophages, CEV1, and determination of its potential to reduce *Escherichia coli* O157:H7 levels in sheep. Applied and Environmental Microbiology. 2006; 72: 6405-10.
[31] Pulido RP, Burgos MJG, Galvez A, Lopez RL. Application of bacteriophages in post-harvest control of human pathogenic and food spoiling bacteria. Critical Reviews in Biotechnology. 2016; 36: 851-61.
[32] Sharma M, Patel JR, Conway WS, Ferguson S, Sulakvelidze A. Effectiveness of bacteriophages in reducing *Escherichia coli* O157:H7 on fresh-cut cantaloupes and lettuce. Journal of Food Protection.2009; 72: 1481-85.
[33] Mukhopadhyay S, Ramaswamy R. Application of emerging technologies to control *Salmonella* in foods: A review. Food Research International. 2012; 45: 666-77.

[34] Hungaro HM, Mendonça RCS, Gouvea DM, Vanetti MCD, de Oliveira-Pinto CL. Use of bacteriophages to reduce *Salmonella* in chicken skin in comparison with chemical agents. Food Research International. 2013; 52: 75-81.
[35] Akhtar M, Viazis S, Diez-Gonzalez F. Isolation, identification and characterization of lytic, wide host range bacteriophages from waste effluents against *Salmonella enterica* serovars. Food Control. 2014; 38: 67-74.
[36] Coffey B, Mills S, Coffey A, McAuliffe O,Ross RP. Phage and their lysins as biocontrol agents for food safety applications. Annual Review of Food Science and Technology. 2010; 1: 449-68.
[37] Higgins JP, Higgins SE, Guenther KL, Huff W, Donoghue AM, Donoghue DJ, Hargis BM. Use of specific bacteriohage treatment to reduce *Salmonella* in poultry products. Poultry Science. 2005; 19: 1141-45.
[38] Spricigo DA, Bardina C, Cortes P, Llagostera M. Use of a bacteriohage cocktail to control *Salmonella* in food and the food industry. International Journal of Food Microbiology. 2013; 165; 169-74.
[39] Wong CL, Sieo CC, Tan WS, Abdullah N, Hair-Bejo M, Abu J, Ho YW. Evaluation of a lytic bacteriophage, ϕst1, for biocontrol *Salmonella enteric*a serovar Typhimurium in chickens. International Journal of Food Microbiology. 2014; 172: 92-101.
[40] Galvez A, Abriouel H, Benomar N, Lucas R. Microbial antagonists to food-borne pathogens and biocontrol. Current Opinion in Biotechnology. 2010; 21; 142-48.
[41] Kim JW, Siletzky RM, Kathariou S. Host ranges of *Listeria* specific bacteriophages from the turkey processing plant environment in the United States. Applied and Environmental Microbiology. 2008; 74: 6623-30.
[42] Klumpp J, Loessner MJ. *Listeria* phages. Bacteriophage. 2013; 3:3, e26861.
[43] Bigot B, Lee WJ, Mclntyre L,Wilson T, Hudson JA, Billington C, Heinemann JA. Control of *Listeria monocytogenes* growth in a ready-to-eat poultry product using a bacteriophage. Food Microbiology. 2011; 28: 1448-52.
[44] Guenther S, Loessner MJ. Bacteriophage biocontrol of *Listeria monocytogenes* on soft ripened white mold and red-smear cheeses. Bacteriophage. 2011; 1:2, 94-100.
[45] Rossi LPR, Almeida CC, Lopes LS, Figueiredo ACL, Ramos MPP, Almeida PF. Occurrence of *Listeria* spp. in Brazilian fresh sausage and control of *Listeria monocytogenes* using bacteriophage P100. Food Control. 2011; 22: 954-58.
[46] Soni KA, Desai M, Aladunjoye A, Skrobot F, Nannapaneni R. Reduction of Listeria monocytogenesin queso fresco cheese by a combination of listericidal and listeriostatic GRAS antimicrobials. International Journal of Food Microbiology. 2012; 155: 82-88.
[47] Fischer S, Kittler S, Klein G, Glünder G. Impact of a single phage and a phage cocktail application in broilers on reduction of *Campylobacter jejuni* and development of resistance. Plos One. 2013; 8: e78543.
[48] Kazi M, Annapure US. Bacteriophage biocontrol of foodborne pathogens. Journal of Food Science and Technology. 2016; 53: 1355-62.
[49] Firlieyanti AS, Connerton PL, Connerton IF. *Campylobacters* and their bacteriophages from chicken liver: The prospect for phage biocontrol. International Journal of Food Microbiology. 2016; 237: 121-127.
[50] Atterbury RJ, Dillon E, Swift C, Connerton PL, Frost JA, Dodd CER, Rees CED, Connerton IF. Correlation of *Campylobacter* bacteriophage with reduced presence of hosts in broiler chicken ceca. Applied and Environmental Microbiology. 2005; 71; 4885-87.
[51] Carvalho CM, Gannon BW, Halfhide DE, Santos SB, Hayes CM, Roe JM, Azeredo J. The in vivo efficacy of two administration routes of a phage cocktail to reduce numbers of *Campylobacter coli* and *Campylobacter jejuni* in chickens. BMC Microbiology. 2010; 10: 232.
[52] Ghareeb K, Awad WA, Mohnl M, Schatzmayr G, Böhm J. Control strategies for *Campylobacter* infection in poultry production. World's Poultry Science Journal. 2013; 69: 57-76.
[53] Orquera S, Hertwig S, Alter T, Hammer JA, Jirova A, Gölz G. Development of transient phage resistance in *Campylobacter coli* against the group II phage CP84. Berl Münch Tierärztl Wochenschr. 2015; 128: Heft 3/4.
[54] Atterbury RJ, Connerton PL, Dodd CER, Rees CED, Connerton IF. Isolation and characterization of *Campylobacter* bacteriophages from retail poultry. Appllied and Environmental Microbiology. 2003; 69; 4511-18.
[55] Carrillo CL, Atterbury RJ, El-Shibiny A, Connerton PL, Dillon E, Scot A, Connerton IF. Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. Applied and Environmental Microbiology. 2005; 71: 6554-63.
[56] Bueno E, Garcia P, Martinez B, Rodriguez A. Phage inactivation of *Stapylococcus aureus* in fresh and hard type cheeses. International Journal of Food Microbiology. 2012; 158: 23-37.
[57] Garcia P, Madera C, Martinez B, Rodriguez A. Biocontrol of *Staphylococcus aureus* in curd manufacturing processes using bacteriophages. International Dairy Journal. 2007; 17; 1232-39.
[58] Haddad LE, Roy JP, Khalil GE, St-Gelais D, Champagne CP.Efficacy of two *Staphylococcus aureus* phage cocktails in cheese production. International journal of Food Microbiology. 2016; 217; 7-13.
[59] Tabla R, Martinez B, Rebollo JE, Gonzalez J, Ramirez MR, Roa I, Rodriguez A, Garcia P. Bacteriophage performance against *Staphylococus aureus* in milk is improved by hih hydrostatic pressure treatments. International Journal of Food Microbiology. 2012; 156; 209-13.

Conventional and non-conventional lipid modifications: a review

K.R.R. Godoi, **D. Barrera-Arellano** and **A.P.B. Ribeiro**

Department of Food Technology, School of Food Engineering, University of Campinas, Campinas, Brazil

Conventional lipid modification processes are able to redesign oils and fats depending on the desired functionality profile and the intrinsic characteristics of the raw materials by chemical modification of the fatty acids (hydrogenation); by reversing the ester linkage (hydrolysis); by physically separating triacylglycerols from glycerol with distinct melting points (fractionation) and rearrangement of fatty acids in the triacylglycerols main chain (interesterification). Although these processes are technologically functional, the substitution of fats in food products is a challenge because is difficult to obtain the satisfactory characteristics when there is no trans fatty acids and with reduction of saturated fatty acids. Thus, organogels technology appears with a new range of lipid structures capable of forming a three-dimensional network that immobilizes liquid oil, resulting in lipid bases with distinct macroscopic characteristics, rich in unsaturated fatty acids that shows potential for substitution of the technical fats obtained by the conventional processes of lipid modification, aiming at industrial products of better nutritional profile.

Keywords: Lipid modification; Organogel; Structuring lipids

1. Introduction

The imbalance in lipoprotein metabolism caused by dietary habits with high intakes of saturated and trans fats and cholesterol causes increased concentration of total cholesterol and low density lipoprotein (LDL*),* reducing the fractions of high density lipoprotein *(*HDL), affecting the LDL/HDL ratio, which favors the onset of cardiovascular diseases. Conventional lipid modification processes are able to redesign oils and fats depending on the desired functionality profile and the intrinsic characteristics of the raw materials by chemical modification of the fatty acids (hydrogenation); by reversing the ester linkage (hydrolysis); by physically separating triacylglycerols (TAG) from glycerol with distinct melting points (fractionation) and rearrangement of fatty acids in the TAG main chain (interesterification). Although these processes are technologically functional, the substitution of fats in food products is a challenge, since technologically satisfactory crystallization and consistency properties are difficult to obtain when there is no trans fatty acids and reduction of saturated fatty acids. As a consequence, public policies that directed the food industry to change the lipid raw materials conventionally employed, aiming for greater consumer health, were implemented. Thus, the technology of organogels appears with a new range of lipid structures capable of forming a three-dimensional network that immobilizes liquid oil, resulting in lipid bases with distinct macroscopic characteristics and obtained with raw materials free of trans fatty acids and rich in unsaturated fatty acids. Organogels, viscoelastic materials composed of structuring agents and an apolar liquid phase, show the potential for substitution of the technical fats obtained by the conventional processes of lipid modification, aiming at industrial products of better nutritional profile.

2. Lipids

The term lipid is derived from the Greek *lipos*, which means fat. However, a number of compounds can be accommodated in this group: oils (mixtures of TAGs in liquid form at room temperature), fats (TAG blends in solid form at room temperature), waxes (esters formed by fatty acids with long-chained alcohols), as well as soaps, steroids, detergents and bile salts [1].

Lipid-forming fatty acids are hydrocarbon derivatives and have a very low oxidation state, similar to the hydrocarbons present in fossil fuels, which means that fatty acids are highly reduced. In addition, they may have chains of 4 to 36 carbons, saturated or unsaturated. Frequently occurring fatty acids in nature have even numbers of carbon atoms in an unbranched chain containing from 12 to 24 carbons. Likewise, in the unsaturated fatty acids, the double bond is generally located at carbons 9 and 10 and are separated by methylene groups, that is, for the most part, unconjugated double bonds occur [2].

According to the primordial chemical structure of lipids, these can be classified as acylglycerols, which have fatty acids esterified to a glycerol molecule. The number of esterified fatty acids classifies acylglycerol in monoacylglycerol (one esterified fatty acid), diacylglycerol (two esterified fatty acids) and TAG (three esterified fatty acids). Natural fats, such as vegetable oils and animal fat, correspond to complex mixtures of simple and mixed TAGs containing a variety of fatty acids that differ in chain length and degree of saturation [2].

From a dietary and nutritional point of view, edible oils and fats are essential nutrients in the human diet, playing a vital role through the delivery of essential fatty acids and energy. In addition to the nutritional qualities, oils and fats provide consistency and melting characteristics specific to the products that contain them as well as acting as a mean of heat transfer during the frying process and as carriers of fat-soluble vitamins and aroma [3].

3. Saturated fats

All humans need a lipid source in their diet. However, the increasing incidence of cardiovascular diseases in the last century has led to a great search for the risk factors related to its development. Thus, several studies have found that the relatively high intake of saturated fat (approximately 17% of total energy) is an important contributor to the high incidence of coronary heart disease [4]. Unbalance in lipoprotein metabolism and dietary habits are major risk factors for cardiovascular diseases. Western diets, with high concentrations of saturated fats and cholesterol, increase the concentration of total cholesterol and low-density lipoprotein (LDL). The activity of the hepatic LDL receptor is generally the main factor controlling plasma LDL, dietary cholesterol and saturated fats, since they suppress this receptor by increasing plasma cholesterol concentrations[1,5].

Saturated fatty acids are naturally present in oils and fats of plant and animal origin, and may produce interesterified or fractionated fats [6,7]. However, when this saturated fat is replaced by a source rich in polyunsaturated fatty acids, a reduction in the plasma cholesterol concentration associated with LDL also occurs, as well as anti-inflammatory actions on the vascular cells, since these fatty acids inhibit the expression of proinflammatory endothelial proteins [4,8].

4. Trans fats

The trans fatty acids (TFA) are geometric and positional isomers of natural unsaturated fatty acids. In this configuration, two hydrogen atoms attached to the carbon atoms forming the double bond are located on opposite sides of the carbon chain, forming a linear and more rigid molecule. Because of their structural characteristics, trans fatty acids have a higher melting point than when compared with their corresponding *cis* isomer [9].

TFAs are naturally present in fats from ruminant animals as a result of the biohydrogenation process in the microbial flora of the rumen. However, TFAs also originate from partial catalytic hydrogenation of vegetable or marine oils. About 90% of TFA in food derives from this process. Hydrogenation is carried out with the aim of modifying the composition, structure and consistency of an oil. This results in the reduction of the degree of oil unsaturation and increase of its melting point, associated with the increase in the oxidative stability and functionality of the semisolid fractions produced [10].

The TFAs are included among dietary lipids that act as risk factors for coronary artery disease, modulating the synthesis of cholesterol and its fractions and acting on eicosanoids. Several studies have suggested a direct relationship between TFA and increased risk of vascular diseases [5,10–12]. The excess of TFA, from the biological point of view, favors the production of eicosanoids without biological activity, since it has priority of desaturases enzymes [1]. In contrast to all other fatty acids, the trans isomers imply a decrease in high-density lipoprotein (HDL) and an increase in LDL cholesterol, affecting the LDL/HDL ratio unfavorably compared with the modification caused only by saturated fatty acids [13].

5. Regulatory aspects

Due to the harmful effects of TFAs and saturated health, several actions have taken place from both Health Regulatory Agencies and Societies responsible for the elaboration of Nutrition Guidelines, in order to recommend the reduction of consumption of these fatty acids by the world population. Thus, public policies that directed the food industry to change the lipid raw materials conventionally employed, aiming for greater consumer health, were implemented [8].

Regarding international bodies, the Food and Drug Administration (FDA) requires that trans fats be declared in the nutritional information on the label of industrialized products since 2006. Recently, in June 2015, a new resolution called for the complete removal of trans fats in processed foods. This measure was taken aiming to reduce the incidence of cardiovascular disease and prevent thousands of fatal heart attacks that occur every year in the United States of America. In addition, the TFAs were also excluded from the GRAS ("generally recognized as safe") classification food safety indicator for human consumption [14].

As a precautionary measure, the FDA has also developed consumer guidelines recommending the choice of non-fat trans and reduced in saturated fat food products. The recommendation also warns about the minimum levels of TFA, since products with contents less than 0.5g of TFA in the portion do not have this component declared in the nutritional information. Therefore, it is recommended to check the listing of ingredients and if there is presence of partially hydrogenated fat, rich in TFA [14].

In Europe, however, the body responsible for food regulations is the European Food Safety Authority, which has warned about the increased risk of death from cardiovascular disease when daily intake of TFA exceeds 2% of total energy value. However, Europe does not have standard legislations for the whole continent – each country has the authority to set limits and recommendations. Possible approaches for European countries to limit the levels of TFA in food can be divided into legislative actions and voluntary measures. Legislative actions define limits (either at the level of ingredients or in the final product) or recommendations on the consumption of TFA in nutritional information and was adopted by Austria, Denmark, Latvia and Hungary. In other countries, there is voluntary inclusion of the trans fat

content in nutrition information, with Belgium, Denmark, the Netherlands, Poland, the United Kingdom and Greece are making their own regulations; While Bulgaria, Malta, Slovakia, the United Kingdom and Finland included dietary recommendations and Estonia changed the criteria for producing food products. Studies have noted that legislative actions were more effective in eliminating TFA in food than voluntary measures [15].

From these considerations, the field of knowledge in lipid technology has sought alternatives to reduce the amount of saturated and trans fats in foods, as well as reducing the caloric intake associated with the lipid content in processed products.

6. Lipid modification processes

Most natural oils and fats have limited application in their unaltered forms, imposed by their particular composition in fatty acids and TAGs. However, in the last decades there has been an increasing interest in the technology of modification of oils and fats. This trend can be attributed mainly to the fact that these materials are obtained from natural sources and are used as important raw materials for the food, chemical and pharmaceutical industries. Therefore, the industrial sector of oils and fats has developed several processes to alter the composition of TAG mixtures [16].

6.1 Conventional processes

In conventional lipid modification processes the basic structure of oils and fats can be redesigned depending on the desired functionality profile and the intrinsic characteristics of the raw materials by chemical modification of the fatty acids (hydrogenation); by reversing the ester linkage (hydrolysis), by physical separation of phases of fatty acids (TAG) from glycerol with distinct melting points (fractionation) and rearrangement of fatty acids in the TAG main chain (interesterification) [3].

Hydrogenation is a process of modification of oils aimed at obtaining fatty bases for the formulation of several food products. Partial hydrogenation modifies the plasticity of the lipid base and generates trans fatty acids. The reaction occurs at the active sites of the catalyst, usually nickel, where the gaseous hydrogen comes in contact with the double bonds of the unsaturated fatty acids present in the oil or fat, giving rise to saturation or isomerization reactions. The double bonds can be repositioned (position isomerization) or modified from the *cis* configuration to a thermodynamically more stable form, by the trans-isomerization mechanism [16]. In turn, total hydrogenation promotes the saturation of all the double bonds of the fatty acids of the starting raw material without forming trans isomers [17].

Interesterification is a process of modification of oils and fats that promotes a redistribution of fatty acids in the chains of glycerol. The reaction begins when the catalyst is added to the oil or fat, promoting the cleavage of the TAG ester bonds. The fatty acids in the free form are re-esterified intra- or intermolecularly, usually resulting in products with distinct melting and crystallization characteristics of the original raw materials [18]. Although interesterified fats are free from trans isomers, fatty acid distribution may occur at the 1, 2 and 3 positions of the glycerol molecule, whose alterations may induce an increased risk of developing cardiovascular diseases [5].

Data from the literature show controversial results regarding the action of these fats on the lipid profile, since different types of fatty acids can be used in their formulation. Thus, it is necessary that studies evaluate not only the action of the various saturated fatty acids used, but also the possible metabolic implications resulting from the modification of the position of these fatty acids in the glycerol molecule [8].

The fractionation process, in turn, consists of the thermomechanical separation in two steps: partial crystallization in liquid phase and filtration. In the first step, the molten fat is cooled and maintained at the desired crystallization temperature until partial crystallization of the higher melting point TAG occurs. At the end of the crystallization step, the two phases are separated by filtration to give stearin (solid phase) and olein (liquid phase). The stearin consist of TAGs rich in saturated fatty acids, SSS (saturated saturated-saturated) and SSU (saturated-saturated-unsaturated) types, while olein is TAG-rich UUU (unsaturated-unsaturated) and UUS (unsaturated unsaturated) [3].

Fractionation and the use of *blends*, that is, mixtures of fats with different physical properties, represent alternatives for obtaining fatty bases with physical properties and plasticity suitable for use in several products, although with limited potential by the chemical composition of the raw materials themselves [19].

Although the processes of interesterification, fractionation and mixing are very technologically functional, the substitution of partially hydrogenated or interesterified fats in food products is currently a challenge, since appropriate characteristics of crystallization and consistency are difficult to obtain when there is absence of trans fatty acids and saturated fatty acids [20].

6.2 Lipids structuration

In recent years, interest in the influence of specific additives or minor components in oils and fats on the physical properties of fatty systems has significantly increased [21]. Modification of physical properties of lipid matrices has been a strategic subject for the processing of high-fat foods, aiming at product suitability, cost reduction, quality improvement and increased applicability and stability of different lipid raw materials [21–23]. Different materials have

been evaluated, such as trisaturated TAGs, free fatty acids, partial acylglycerols (monoacylglycerols and diacylglycerols), phospholipids, phytosterols and emulsifiers [24].

Generally, the structuring of oils and fats is carried out through the organization of the TAG in the initial stages of the solidification process forming a network of small crystals in the oil. The size and shape of these crystals can be controlled by the shear rate and degree of cooling, determining the mechanical properties of the crystalline lattice [25].

The range of publications available to date indicates that the various components, either natural or added to the lipid matrix, act at the molecular or sub-micron level on the overall structuring process, involving nucleation, crystalline growth, morphology, thermal behavior and polymorphic stability. Similarly, the effects of these modifiers at the macroscopic level, such as visual appearance, melting profiles, rheology and consistency, have also been the subject of further studies [24].

In this context, in addition to the conventional structuring of TAG, studies on a new range of lipid structures have shown several effective compounds for the formation of a three-dimensional network able to immobilize the liquid oil, forming lipid bases with distinct macroscopic characteristics as an alternative to the conventional structuring agents rich in saturated fatty acids or trans fatty acids. Products of this new technology have been denominated organogels or oleogels [26].

7. Organogels theory

By definition, organogels are semi-solid systems, where the oil phase is immobilized by a self-sustaining three-dimensional network formed by the structuring agents [22,24–27].

The organogel-forming structuring agents are characterized as lipid materials capable of capturing a large amount of liquid oil, and can be classified into two groups: self-assembling systems (self-assembly) or crystalline particle systems. Both structuring agents act as building blocks forming three-dimensional networks in liquid or semi-solid oils. The size of the structuring agents and their interactions may determine the final structure of the product and its characteristics (Figure 1) [25,27]. The structuring can be achieved by dispersing the structuring agents in the continuous phase. These structuring agents may be macromolecules, such as proteins or polymers, or low molecular weight compounds such as fatty acids, fatty alcohols or TAGs. The interaction between them can be of different natures through covalent bonds, electrostatic forces, hydrogen bridges or Van der Waals forces. Generally, a small amount of structuring interacting with the continuous phase is sufficient for forming a network [25].

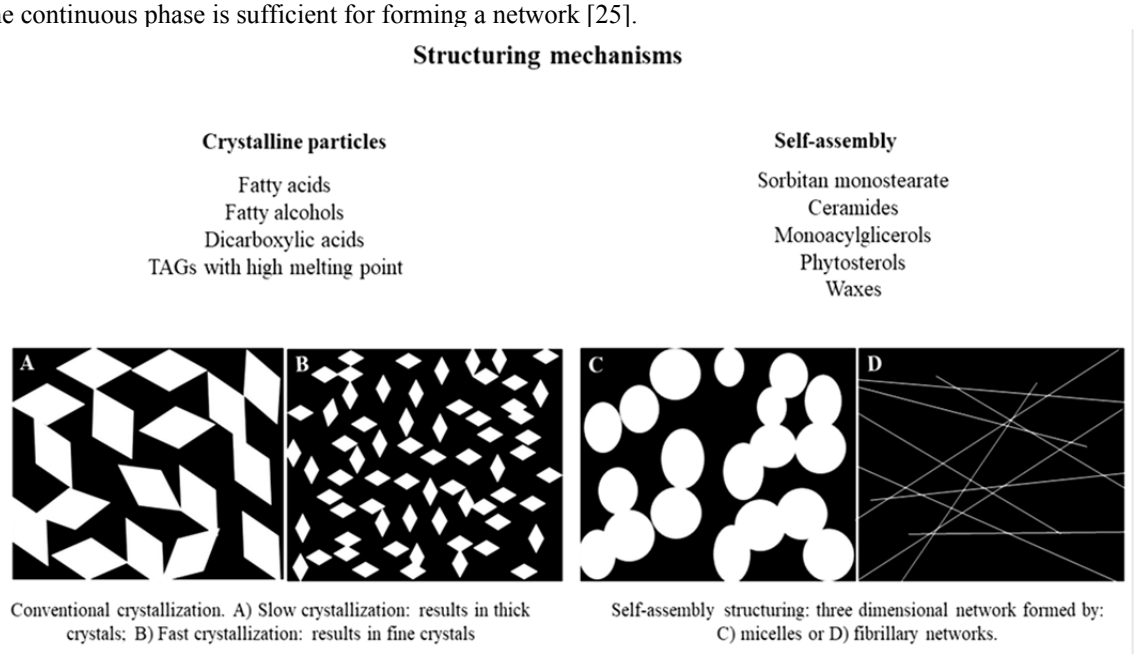


Fig. 1 Different structuring mechanisms obtained in organogels.

The structuring agents of the crystalline particles type form colloidal crystalline lattices inducing the crystallization of the TAGs, which when subjected to cooling, limit the solubility of the higher melting molecules leading to the nucleation events. In this system, the crystals grow and interact with each other through non-covalent forces forming a continuous three-dimensional network. After completion of the crystallization, the crystals aggregate forming larger and larger agglomerates from weak bonds, and give rise to the final macroscopic characteristics [25].

The self-sustaining promoter structures may be represented by macromolecules, low molecular weight compounds such as fatty acids and fatty alcohols, mixtures of phytosterols-oryzanols, sorbitan monostearate, sorbitan tristearate, mixtures of lecithin and waxes. Using these agents, liquid or semi-solid vegetable oils can be structured as gels, forming

continuous networks, micelles or fibrillary networks from aggregates of micelles, developing inverse bilayer structures in the form of rods, characterizing the materials recognized as organogels or oleogels [21,25].

When the structuring agents used are of lipid origin, the formation of the gels occurs through an extension of the conventional crystallization process, the type, morphology and mode of growth of the crystals being distinct from the conventional crystallization mode, resulting in networks through the oil-structuring bonding capacity [28].

In gels added with only one component, as in the case of waxes in oil, this structuring intensifies linearly to the length of the acyl chain, which suggests an alignment of the structures forming a network stabilized by intermolecular hydrogen bonds. In mixed systems (lipid and emulsifying materials, for example), gelation is influenced by changes in the microstructure induced by heterogeneous nucleation, high crystallization kinetics, polymorphic habit modification and higher mesh density. Such changes in crystallization characteristics result in distinct networks capable of immobilizing the continuous lipid phase and originating the organogels [28].

Depending on the type of structuring element and the connections made for network formation, organogels with different properties can be formed affecting the characteristics of the product in which it is applied. The size and shape of the crystals formed by TAG can be controlled through cooling and shear and the interactions determine the mechanical properties of the network. When a large amount of structuring agent is used, a firm and dense network can be formed according to the desired profile, as well as the fluid texture organogels, whose production is made with the interaction of high and low melting TAGs [25].

The use of diacylglycerols and monoacylglycerols results in softer organogels, and, regardless of the compound, the longer the chain length, the greater the firmness of the gel obtained. Waxes, for example, have long chains and, a small amount of the material is already capable of forming a three-dimensional self-sustaining network. In addition, emulsifiers such as sorbitan monostearate, lecithin and sorbitan tristearate produce viscous solutions in edible oils, and viscosity increases with increasing concentrations of structuring agents [25].

Organogels demonstrate the potential to improve the physical characteristics of a product for industrial use without increasing the content of saturated fatty acids or trans fatty acids, making possible the development of products with reduced levels of saturated fatty acids, maintaining their sensory characteristics of texture and taste [21].

In addition, another interesting application of the organogels relates to the inhibition of the migration of oil into food products containing solid and liquid lipid phases such as in chocolates. It is expected that, through the immobilization of the liquid fraction, the mobility of the TAG is reduced, avoiding the modification of polymorphic forms, as occurs in the phenomenon of fat bloom, in bars and chocolate fillings [22].

7.1 Organogels rheology

Rheology is the branch of physics that studies the properties of flow and deformation of matter. The formulations of some materials cannot be differentiated into solids or liquids with clarity, so that the rheological property of interest in these cases is viscoelasticity, which occurs in the case of organogels, which are difficult to characterize materials, as they have solid and liquid properties at the same time. The determination of the rheological parameters can help in the characterization of the materials produced and in the understanding of the physico-chemical nature, besides allowing the understanding of the effects of the components of the formulation and the temperature used in the stability of the material [29].

Analysis of rheological behavior can be performed through parallel plate or cone-plate systems, with a small amount of sample deposited between the plates, one of which rotates and the other is fixed. Such a configuration enables to control the rotation and the calculation of the shear rate. The material then exerts a force of resistance to movement, which is measured as torque by the equipment [30].

Fluids may have newtonian, pseudoplastic, dilatant or plastic characteristics according to their behavior in relation to deformation or flow (Figure 2) [31].

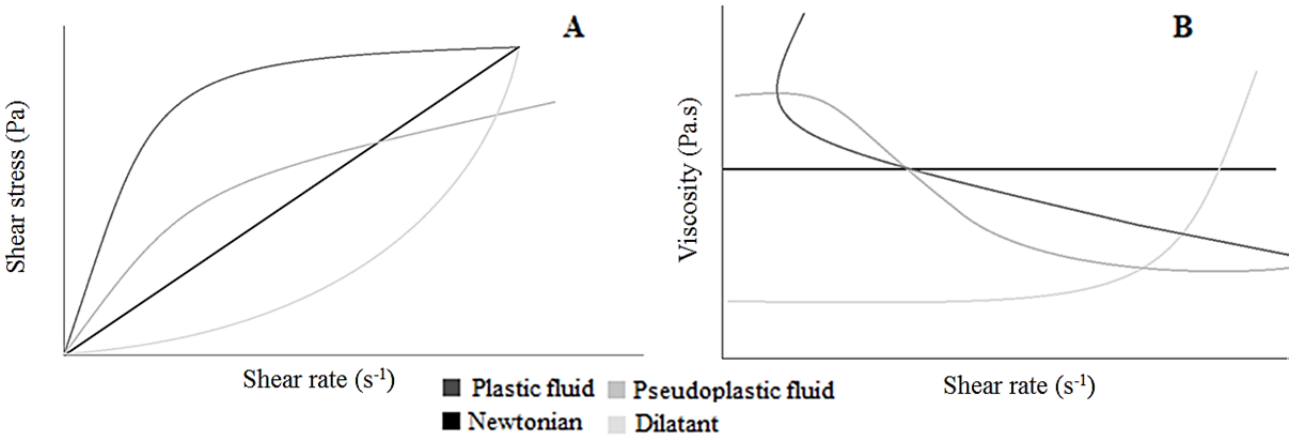


Fig. 2 Fluids classification through the behavior in relation to shear rate and shear stress (A) and shear rate and viscosity (B).

In a study performed by WRIGHT; MARANGONI [32], the profile of a vegetable-oil-based organogel, added with ricinoleic acid, was evaluated through the characterization of crystallization kinetics, microstructure, X-ray diffraction and the rheology of the gel . With the latter, it was possible to classify and identify the influence of various concentrations of ricinoleic acid on the behavior of the gel, which, between 1 and 5% (m:m) of concentration of the structuring agent, allowed to characterize the organogels as viscoelastic. In the study, the authors observed that changes in the rheological properties of the gel are more dependent on the structuring agent concentration than on the analysis temperature.

Lupi et al. [33] evaluated the influence of different types of oil phase (medium chain TAG and long chain TAG) on the process of gelling organogels with beeswax. Results showed that the increase in wax concentration led to higher values of storage and loss modulus (G', G") and complex modulus (*) of the organogels, which is associated with the net force formed between the oil and the structurant, increasing the strength and firmness of the gels.

Ramírez-Gómez et al. [34] produced high oleic safflower oil (OCAO) organogels containing candelilla wax (CC) and fully hydrogenated soybean oil (OSTH) as structurants in the proportions 1:15, 1:10, 2:15, 1:5, 3:10 , 2:5, 3:5 (m:m), or isolated, and evaluated them according to parameters of thermal behavior, polymorphism, microstructure and rheology. The rheological characteristics observed in systems consisting of 1% to 3% of isolated CC and 5% to 15% of isolated OSTH in OCAO classified these gels as viscoelastic. In addition, organogels with smaller crystals (high concentrations of CC) presented increase of G' compared with those structured with large spherulites (OSTH) while gels structured with interlaced platelets (2-3% CC) practically did not recover. This recovery effect to the initial state is desired for application in foods such as whipped cream and puff pastry.

8. Materials of interest for composition of organogels

8.1 Fully hydrogenated palm oil

Among the possible modifications in edible oils, total hydrogenation eliminates the double bonds of unsaturated fatty acids, forming hardfats or fully hydrogenated vegetable oils, materials used as crystallization additives in vegetable oils that may be capable of altering the polymorphic habit and crystallization behavior of the lipid matrix [17].

Hardfats play important role in the structuring of TAGs due to their insolubility, or limited solubility, and the ability to form a solid crystal lattice. The hardfat origin may affect its composition, but, in general, all the hardfats have similar melting characteristics, with a high melting point and a crystalline matrix that withstands high temperatures [24,35].

The hardfat of palm oil is characterized almost exclusively by palmitic (C16:0) and stearic (C18:0) acids, with approximate concentrations of 44 and 54%, respectively [3]. High-melting TAGs, present in fully hydrogenated palm oil, cause increased crystallization velocity, since the fatty acid chains present are average (C16:0 and C18:0) and have a high melting point, near at 65°C [35]. The presence of trisaturated TAGs and the predominance of fatty acids of higher chains enable to reduce the induction time of crystallization by acting as preferential crystallization nuclei. Hardfats obtained from rapeseed, soybean and palm oils are usually used in concentrations up to 5% in addition to the continuous lipid phase to increase the rate of crystallization [17].

Oliveira et al. [36] evaluated the behavior of fully hydrogenated palm, soybean, cotton and crambe oils at various concentrations added isolately in palm oil. As a result, they observed that the addition of these hardfats reduced the induction time of crystallization in all of the blends, while increasing the thermal resistance of the obtained lipid bases.

Santos [37] evaluated the performance of the palm oil hardfat at the concentrations of 1, 3 and 5% and of soy lecithin (1%) in the crystallization profile of refined palm oil and mixtures of palm oil with palm olein (25, 50 and 75%) and observed that the palm hardfat reduced the induction period with consequent acceleration of the crystallization rate and from the synergism between soybean lecithin and palm hardfat in the consistency, both acting positively as structuring agents.

The palm hardfat is allowed for use as a structuring agent in foods, however, due to its composition of saturated fatty acids (palmitic and stearic acid), it should be added in small proportions in such a way not to increase the saturated fatty acids content in the organogel and in the final product.

8.2 Candelilla wax

Vegetable waxes are defined as a mixture of long chain non-polar compounds, including primarily fatty acid esters with long chain alcohols and only one functional group [38,39]. Waxes may be natural or synthetic, and the natural waxes are found on the surface of plants, protecting them, and they are approved for use in food as ingredients or additives according to the Food and Drug Administration [40].

The candelilla wax*, Euphorbia cerifera,* of the Euphorbiaceae family, originates from shrubs in northern Mexico and southwestern United States of America. The shrub is rarely attacked by pests and diseases and much of its production is directed to the market. The wax present in candelilla provides moisture barrier, being of paramount importance to prevent the dehydration of xerophytic plants. Their composition has 49-50% of *n*-alkanes having 29-33 carbon atoms, 20-29% high molecular weight esters, 12-14% sterols and alcohols and 7-9% free fatty acids [41,42].

Because it is a vegetal product, the wax extracted from the candelilla can present variations due to the seasonality and characteristics of the planting site. A 1995 study compared candelilla waxes grown in Texas (USA), Arizona (USA), Chihuahua (Mexico) and Durango (Mexico). The waxes showed diverse composition in hydrocarbons, being the biggest difference observed in the plant harvested in Arizona. In general, waxes have from 45 to 52% hydrocarbons predominating with alkanes and alkenes; and the remaining material is composed of esters, alcohols and resins [42].

The presence of *n*-alkanes in candelilla wax is what guarantees its structuring property, since these compounds act as self-assembly agents in several apolar arrays, forming crystalline networks with chips format. These chips are joined and form the three-dimensional network that confers the macroscopic characteristics of the obtained organogels [43].

Candelila wax is mainly used in the cosmetics industry for the production of lip protectors and lotions in bars and in the paint industry for the manufacture of varnishes. In addition, it has been used to replace carnauba wax and beeswax in different food systems, due to its composition that allows the development of edible organogels through dispersions of candelilla wax in vegetable oils [39,44].

Vegetable waxes, mainly candelilla and carnauba wax, are promising structuring agents for liquid oils, as they are commercially available and result in low cost, considering the amount needed for gelation. The three-dimensional network formed by candelilla wax presents small crystals with low spatial distortion, larger surface area and small pores capable of immobilizing the oil phase. In addition to the concentration and the particularities of the waxes, external factors, such as shear and cooling rate, affect the formation of the network and the ability to bond between wax and oil. However, due to the versatility and thermal reversibility of organogels obtained with wax, its use has been exploited in different applications such as fat reduction in ice cream, margarine and other food systems [20,45,46].

In recent studies on the structuring property of candelilla wax, Toro-Vazquez et al.[44] evaluated the incorporation of about 1% of wax in safflower oil, analyzing later the thermodynamic properties of the mixture. Hwang et al. [47] observed that candelilla wax associated with rice bran wax showed good structuring properties, further revealing that small amounts of vegetable waxes can replace lipid raw materials with high trans or saturated fat contents. In recent studies, Hwang et al. [48] analyzed the formation of organogels in soybean oil using sunflower wax and concluded that the esters present in the wax were important contributing factors for the construction of the formed three-dimensional network.

Rocha et al. [39] compared the effect of adding sugarcane wax and candelilla wax to 4% soybean oil concentration. Both showed the capacity to form organogels, however, the rheological properties and the compression/extrusion analysis showed that the organogel formed by candelilla wax had higher mechanical resistance even under shear, which could be proved by microscopy under polarized light in which the organogel presented a more organized network. The authors attributed the results to the differentiated chemical composition of the waxes used, especially in relation to chain size and the presence of alcohols that can affect the interaction of the structurant with the liquid phase.

Consumption of candelilla wax is allowed within the limits of the legislations, because its ingestion is considered of low toxicity since the wax compounds are absorbed through normal metabolic pathways, without there being accumulation in the body. Hence, recent studies have indicated that candelilla wax is safe for human consumption [49].

8.3 Sorbitan monostearate

Sorbitan monostearate (SMS) is a non-ionic hydrophobic surfactant, produced by esterification of sorbitol with stearic acid. SMS is characterized as a granular solid that is dispersed in organic phases by heating to 60ºC, resulting in an opaque, whitish suspension with smooth textured gel. In cooling, the affinity between the solvent and the SMS decreases allowing the self-assembly mechanism of the system, in which the molecules of the emulsifier aggregate forming a three-dimensional network that immobilizes the solvent and is therefore able to form organogels in a variety of vegetable oils. In microscopic analysis performed by MURDAN; GREGORIADIS; FLORENCE [50] the SMS presented aggregates of tubular structures, cylindrical and elongated.

The addition of SMS in organogels as structuring agent has been made in formulations for the pharmaceutical, cosmetic and food industry. For food purposes, the use of SMS is allowed by the FDA as a food additive with a maximum content corresponding to 1% of the weight of the finished product and guarantees greater thermodynamic stability for long periods [14,21].

Sorbitan esters are generally composed of a mixture of fatty acids of different chain lengths and sorbitol molecules, as shown in Figure 3. Through this configuration, SMS is considered an emulsifier capable of influencing the delay of the polymorphic transitions in the crystallization of TAG [51]. In initial studies with oils and fats, which present a complex mixture of TAGs, the addition of 5% sorbitan esters to cocoa butter promoted the polymorphic transition from the β-form to the βV form and prevented the transition from βV to βVI, making possible a cocoa butter with presence of polymorph βV, which is of great importance to control the visual defect denominated fat bloom that occurs in chocolates [24,52].

In an in-depth study on the effect of the addition of SMS as a crystallization modifier on chocolate production, MASUCHI [53] observed that with the addition of SMS, even at minimum concentrations (about 0.5% m:m) it was possible to significantly modify the crystallization and microstructure of the cocoa butter, being effective in the modulation of the crystallization and delaying the appearance of fat bloom.

From the mechanism of co-crystallization, SMS acts as a modulator of the crystallization due to the molecular similarity with the TAGs of the cocoa butter, which are composed mainly of palmitic, oleic and stearic acids. When evaluated with continuous lipid phase, after heating to about 60°C, the cooling of the system decreases the solubility of the SMS in organic solvents as the lipid phase, forming gel structure [53,54]

Recently, SAGIRI et al. [55] described the effects of the addition of SMS to the mustard oil. Concentrations of 1 to 22% of SMS were used and with this, it was verified that the minimum required concentration of isolated SMS to immobilize the mustard oil was 17% (m:m), being able to render the system semi-solid. The density of the net formed, observed by microscopy, was proportional to the amount of structuring agent added to the oil, that is, the higher the SMS content, the denser the network. Such modification also ensured an increase in the mechanical stability of the organogels produced.

STAHL [56] analyzed the effect of incorporation of SMS associated with hardfat of soybean oil in blends of linseed oils with palm oil in different proportions. Both structuring agents, at 3% concentration, demonstrated the ability to structure lipid matrices with high levels of unsaturated fatty acids, with positive effects on solids content, crystallization kinetics, consistency, thermal behavior, microstructure and polymorphism. Such an effect can be attributed to the presence of stearic acid in the SMS which could favor its co-crystallization with TAG by incorporation on the surface of the crystals, limiting subsequent agglomerations.

OLIVEIRA et al. [57], in turn, observed the combined effect of structuring agents of the self-assembly type, in this case the SMS, with crystalline particle-type structuring agent, represented by the fully hydrogenated canola oil (*hardfat* of canola oil). In the work, mixtures containing palm oil and canola oil were made in proportions of 100:0; 75:25 and 50:50 (w:w) with subsequent addition of 6% structuring agents (50:50 OCTH:SMS), resulting in increased thermal resistance and consistency of lipid bases, which showed synergism and efficacy of OCTM and SMS. The authors verified that the association of self-assembly and crystalline particles structuring agents leads to an optimization of the lipid structuring process, allowing a reduction in the amount of each structuring element, obtaining real organogels.

The consumption of this structuring agent is allowed according to the National Library of Medicine of the United States of America (NLH), it is considered a non-carcinogenic compound that is not retained by the body and is eliminated through the urine [58].

9. Application of organogels in food

The application of organogels has been studied for some years. In the food industry, organogels have the potential to be applied in order to minimize the migration of oils into multi-component foods such as filled chocolate, margarines, baked goods (such as biscuits and cookies), puff pastry and spreads, in addition to ensuring adequate texture and consistency without the addition of saturated or trans fatty acids [21,22].

In 2013, HWANG et al. [59] have developed organogels of vegetable waxes and soybean oil for application in margarines. The waxes used were candelilla wax, rice wax and sunflower wax, in isolation. The study demonstrated the efficacy of the use of oils rich in unsaturated fatty acids, such as soybean oil, ensuring greater wholesomeness to margarine and spreads obtained with the addition of waxes.

CHAVES [60] produced reduced margarines in saturated fatty acids (from 17% to 36% reduction in final product compared with the conventional) with addition of soybean oil organogels or high oleic sunflower oil containing candelilla wax, interesterified fat and monoacylglycerols. The produced margarines presented greater stability in comparison with the commercial margarines and the use of candelilla wax was essential to obtain this result, since the test with absence of candelilla wax showed oil exudation. In general, the parameters of consistency, spreadability, emulsion stability in relation to temperature fluctuations and hardness were similar to commercial margarines.

In similar study, YILMAZ; OGUTCU [61] produced organogels from hazelnut oil and olive oil using bee and sunflower waxes as structuring agents, also for margarine application. In this case, the authors achieved characteristics very similar to commercial margarine. The sensory evaluation of the final product indicated that about 50% of the testers would possibly buy the product.

Also using waxes as a structuring agent, ZULIM BOTEGA et el [62] have developed organogels to replace the lipid fraction in ice cream. Blends containing 10% wax (from candelilla, rice or carnauba) were evaluated with 90% high oleic sunflower oil and glycerol monooleate as emulsifier. In this case, the use of organogels as lipid fraction was able to confer adequate structure to the ice cream, characterizing it as similar to conventional.

The substitution of shortenings by organogels in bakery products has also been performed to reduce the saturated and trans fat content. In studies performed by JANG et al. [63], the lipid fraction of cookies was replaced by organogel from canola oil and candelilla wax, whose hardness was lower than that of conventional shortening at room temperature, as well as the results observed in rheological parameters. When added to the cookies, the organogel product generated less bursting strength, resulting in greater softness, a feature different from that desired for commercial cookies. However, the fatty acid composition indicated that with the substitution of shortening, there was a 92% increase in the content of unsaturated fatty acids.

In a similar study, organogels containing 5% candelilla wax in sunflower oil were also added in cookies resulting in very similar parameters, with softer, less crunchy cookies and with changes in the rheological parameters,

characterizing them as more fluid, and expressive increase of the content of unsaturated fatty acids [64]. In both studies, the cookies sensory analysis test was not performed.

BEMER et al. [65], produced organogels with soybean oil and 10% structurants (rice wax or ethylcellulose) for application in cream cheese. In relation to the conventional product, to that added organogel resulted in a reduction of 90% in the content of saturated fatty acids. The rice wax provided a texture very similar to the commercial cream cheese and, in contrast, the organogel with ethylcellulose showed lower adhesiveness and modulus of elasticity than the commercial parameters. Thus, the study evidences the applicability of organogels containing vegetable wax to the development of healthier products.

Therefore, it is observed that studies on the application of organogels are still scarce, but extremely current and relevant in the area of lipid technology. The development and characterization of organogels are therefore an important technological alternative for obtaining lipid bases with a technological profile suitable for the application in several food, free from trans isomers and with a reduction in saturated fatty acid content.

10. Conclusion

Due to the deleterious effects attributed to the high consumption of saturated and trans fatty acids, organogels present themselves as a potential technological alternative suitable for application in food products, minimizing the migration of oils into multi-component foods, guaranteeing the desired technological characteristics in products such as filled chocolate, margarines, ice cream, spreads, cream cheese and baked goods such as cookies, and puff pastry. These applications, already described in the literature, were able to reduce the content of saturated fatty acids, without the presence of trans fatty acids, demonstrating the wide diversity for the application of organogels in food products according to the characteristics of the obtained systems.

References

[1] R. Curi, J. Procópio, C.K. Myasaka, C. Pompéia, Entendendo a gordura: os ácidos graxos, 1ª, Editora Manole, 2002.

[2] C. Scrimgeour, Chemistry of Fatty Acids, Bailey's Industrial Oil and Fat Products. (2005) 1–43. doi:10.1002/047167849X.bio005.

[3] R. O'Brien, Fats and oils – Formulating and processing for applications, in: Fats and Oils – Formulating and Processing for Applications, CRC Press, New York, 2004: pp. 1–55.

[4] F.E.L. De Lima, T.N. De Menezes, M.P. Tavares, S.C. Szarfarc, R.M. Fisberg, Ácidos Graxos e doenças cardiovasculares: Uma revisão, Revista de Nutrição. 13 (2000) 73–80. doi:10.1590/S1415-52732000000200001.

[5] H.T. Xavier, M.C. Izar, J.R. Faria Neto, M.H. Assad, V.Z. Rocha, A.C. Sposito, F.A. Fonseca, J.E. dos Santos, R.D. Santos, M.C. Bertolami, A.A. Faludi, T.L.R. Martinez, J. Diament, A. Guimarães, N.A. Forti, E. Moriguchi, A.C.P. Chagas, O.R. Coelho, J.A.F. Ramires, V Diretriz Brasileira de Dislipidemias e Prevenção da Aterosclerose, Arquivos Brasileiros de Cardiologia. 101 (2013) 01–22. doi:10.5935/abc.2013S010.

[6] V.L. Chiara, R. Silva, R. Jorge, A.P. Brasil, Ácidos graxos trans: Doenças cardiovasculares e saúde materno-infantil, Revista de Nutricao. 15 (2002) 341–349. doi:10.1590/S1415-52732002000300010.

[7] A.G.V. Costa, J. Bressan, C.M. Sebarense, Ácidos Graxos Trans: Alimentos e Efeitos na Saúde, Archivos Latinoamericanos de Nutrición. 56 (2006) 12–21.

[8] R. Santos, A. Gagliardi, H.T. Xavier, C. Magnoni, R. Cassani, A. Lottenberg, I Diretriz sobre o consumo de Gorduras e Saúde Cardiovascular, Arquivo Brasileiro de Cardiologia. 100 (2013) 1–40. doi:10.5935/abc.2014S004.

[9] D.S. Nichols, K. Sanderson, Chemical and functional properties of food lipids, CRC Press, New York, 2003.

[10] A.P.B. Ribeiro, J.M. Leite, N. De Moura, R. Grimaldi, L.A.G. Gonçalves, Interesterificação química: Alternativa para obtenção de gorduras zero trans, Quimica Nova. 30 (2007) 1295–1300. doi:10.1590/S0100-40422007000500043.

[11] D. Emanuelle, D.L. Viana, M.M. Dantas, M. Emília, Ácidos graxos das séries ômega-3 e ômega-6 e sua utilização no tratamento de doenças cardiovasculares : uma revisão, 5 (2016) 65–83.

[12] A.F. Simão, D.B. Précoma, J.P. de Andrade, H. Correa Filho, J.F.K. Saraiva, G.M.M. de Oliveira, I Cardiovascular Prevention Guideline of the Brazilian Society of Cardiology - Executive Summary, Arquivos Brasileiros de Cardiologia. 102 (2014) 420–431. doi:10.5935/abc.20140067.

[13] P. Wassell, G. Bonwick, C.J. Smith, E. Almiroin-Roig, N.W.G. Young, Towards a multidisciplinary approach to structuring in reduced saturated fat-based systems – a review, International Journal of Food Science and Technology. 45 (2010) 642–655.

[14] Food and Drug Administration, FDA Cuts Trans Fat in Processed Foods, (2015). http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm372915.htm.

[15] D.R. Tobergte, S. Curtis, Report from the commission to the european parliament and the council regarding trans fats in foods and in the overall diet of the Union population, Journal of Chemical Information and Modeling. 53 (2013) 1689–1699. doi:10.1017/CBO9781107415324.004.

[16] B.S. Ghotra, S.D. Dyal, S.S. Narine, Lipid shortenings: A review, Food Research International. 35 (2002) 1015–1048. doi:10.1016/S0963-9969(02)00163-1.

[17] G.M. de Oliveira, A.P.B. Ribeiro, T.G. Kieckbusch, Hard fats improve technological properties of palm oil for applications in fat-based products, LWT - Food Science and Technology. 63 (2015) 1155–1162. doi:10.1016/j.lwt.2015.04.040.

[18] D.R. Erickson, Practical Handbook of Soybean Processing and Utilization, in: David R.Erickson (Ed.), Practical Handbook of Soybean Processing and Utilization, AOCS Press, Champaign, Illinois, 1995: pp. 227–296.

[19] I. Foubert, D. Van De Walle, K. Dewettinck, A.T. Dijkstra, The Lipid Handbook, in: The Lipid Handbook, 3ª Edição, CRC Press, Boca Raton, 2007: pp. 741–508.
[20] J. Reyes-Hernández, E. Dibildox-Alvarado, M.A. Charó-Alonso, J.F. Toro-Vazquez, Physicochemical and rheological properties of crystallized blends containing trans-free and partially hydrogenated soybean oil, JAOCS, Journal of the American Oil Chemists' Society. 84 (2007) 1081–1093. doi:10.1007/s11746-007-1141-6.
[21] M.A. Rogers, Novel structuring strategies for unsaturated fats - Meeting the zero-trans, zero-saturated fat challenge: A review, Food Research International. 42 (2009) 747–753. doi:10.1016/j.foodres.2009.02.024.
[22] N.E. Hughes, A.G. Marangoni, A.J. Wright, M. a. Rogers, J.W.E. Rush, Potential food applications of edible oil organogels, Trends in Food Science & Technology. 20 (2009) 470–480. doi:10.1016/j.tifs.2009.06.002.
[23] A.G. Rogers, Michael A.; Wright, Amanda J.; Marangoni, Oil organogels: the fat of the future?, Soft Matter . 5 (2009) 1594–1596. doi:10.1039/B822008P.
[24] K.W. Smith, K. Bhaggan, G. Talbot, K.F. Van Malssen, Crystallization of fats: Influence of minor components and additives, JAOCS, Journal of the American Oil Chemists' Society. 88 (2011) 1085–1101. doi:10.1007/s11746-011-1819-7.
[25] M. Pernetti, K.F. van Malssen, E. Fl??ter, A. Bot, Structuring of edible oils by alternatives to crystalline fat, Current Opinion in Colloid and Interface Science. 12 (2007) 221–231. doi:10.1016/j.cocis.2007.07.002.
[26] A.G. Marangoni, N. Garti, Edible Oleogels, in: A.G. Marangoni, N. Garti (Eds.), Edible Oleogels, First Edit, AOCS Press, Urbana, Illnois, 2011: pp. 1–18. doi:10.1016/B978-0-9830791-1-8.50007-3.
[27] L.S.K. Dassanayake, D.R. Kodali, S. Ueno, Formation of oleogels based on edible lipid materials, Current Opinion in Colloid and Interface Science. 16 (2011) 432–439. doi:10.1016/j.cocis.2011.05.005.
[28] A.R. Patel, K. Dewettinck, Edible oil structuring: an overview and recent updates, RSC Food Function. (2015) 20–29. doi:10.1039/C5FO01006C.
[29] Barnes, Hutton, Walters, An Introduction to Rheology, 1989. doi:10.1016/0377-0257(89)85015-3.
[30] J.F. Steffe, Rheological Methods in, 1996. doi:10.1016/0260-8774(94)90090-6.
[31] G. Schramm, Reologia e reometria: fundamentos teoricos e praticos, 2ª Edição, Artliber, São Paulo, 2006.
[32] A.J. Wright, A.G. Marangoni, Formation, structure, and rheological properties of ricinelaidic acid-vegetable oil organogels, Journal of the American Oil Chemists' Society. 83 (2006) 497–503. doi:10.1007/s11746-006-1232-9.
[33] F.R. Lupi, V. Greco, N. Baldino, B. de Cindio, P. Fischer, D. Gabriele, The effects of intermolecular interactions on the physical properties of organogels in edible oils, Journal of Colloid and Interface Science. 483 (2016) 154–164. doi:10.1016/j.jcis.2016.08.009.
[34] N.O. Ramírez-Gómez, N.C. Acevedo, J.F. Toro-Vázquez, J.J. Ornelas-Paz, E. Dibildox-Alvarado, J.D. Pérez-Martínez, Phase behavior, structure and rheology of candelilla wax/fully hydrogenated soybean oil mixtures with and without vegetable oil, Food Research International. 89 (2016) 828–837. doi:10.1016/j.foodres.2016.10.025.
[35] S. Norberg, United Kingdom: Woodhead Publishing Limited. 2006, 488 - 516., in: F.D. Gunstone (Ed.), Modifying Lipids Use in Food, Woodhead Publishing Limited, United Kingdom, 2006: pp. 488–516.
[36] G.M. de Oliveira, A.P. Badan Ribeiro, A.O. dos Santos, L.P. Cardoso, T.G. Kieckbusch, Hard fats as additives in palm oil and its relationships to crystallization process and polymorphism, LWT - Food Science and Technology. 63 (2015) 1163–1170. doi:10.1016/j.lwt.2015.04.036.
[37] V. da S. Santos, Estruturação de óleo de palma e emulsões utilizando óleo de palma totalmente hidrogenado e lecitina de soja, 2014.
[38] J. Pokorný, S. Schmidt, Chemical, Biological, and Functional Aspects of Food Lipids, in: Chemical, Biological, and Functional Aspects of Food Lipids, Forth Edit, Taylor and Francis, 2011: pp. 249–269.
[39] J.C.B. Rocha, J.D. Lopes, M.C.N. Mascarenhas, D.B. Arellano, L.M.R. Guerreiro, R.L. da Cunha, Thermal and rheological properties of organogels formed by sugarcane or candelilla wax in soybean oil, Food Research International. 50 (2013) 318–323. doi:10.1016/j.foodres.2012.10.043.
[40] Food and Drug Administration, Everything added to food in the United States (EAFUS), (2012). http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=eafusListing.
[41] J.F. Toro-Vasquez, M.A. Charó-Alonso, J.D. Pérez-Martínez, J.A. Morales-Rueda, Candelilla Wax as an Organogelator for Vegetable Oils—An Alternative to Develop Trans-free Products for the Food Industry, in: A.G. Marangoni, N. Garti (Eds.), Edible Oleogels, AOCS Press, Illinois, 2011: pp. 119–148.
[42] G.A. Scora, M. Ahmed, R.W. Scora, Epicuticular hydrocarbons of candelilla (Euphorbia antisiphylitica) from three different geographical areas, Industrial Crops and Products. 4 (1995) 179–184. doi:10.1016/0926-6690(95)00030-G.
[43] D.J. Abdallah, R.G. Weiss, n -Alkanes Gel n -Alkanes (and Many Other Organic Liquids), Langmuir. 16 (2000) 352–355. doi:10.1021/la990795r.
[44] J.F. Toro-Vazquez, J.A. Morales-Rueda, E. Dibildox-Alvarado, M. Char??-Alonso, M. Alonzo-Macias, M.M. González-Chavez, Thermal and textural properties of organogels developed by candelilla wax in safflower oil, JAOCS, Journal of the American Oil Chemists' Society. 84 (2007) 989–1000. doi:10.1007/s11746-007-1139-0.
[45] J. a Morales-rueda, E. Dibildox-Alvarado, M. a Charó-Alonso, J.F. Toro-Vazquez, Rheological Properties of Candelilla Wax and Dotriacontane Organogels Measured with a True-Gap System, Jaocs. (2009) 765–772. doi:10.1007/s11746-009-1414-3.
[46] H. Si, L.-Z. Cheong, J. Huang, X. Wang, H. Zhang, Physical Properties of Soybean Oleogels and Oil Migration Evaluation in Model Praline System, Journal of the American Oil Chemists' Society. (2016) 1–10. doi:10.1007/s11746-016-2846-1.
[47] H.S. Hwang, S. Kim, M. Singh, J.K. Winkler-Moser, S.X. Liu, Organogel formation of soybean oil with waxes, JAOCS, Journal of the American Oil Chemists' Society. 89 (2012) 639–647. doi:10.1007/s11746-011-1953-2.
[48] H.S. Hwang, S. Kim, K.O. Evans, C. Koga, Y. Lee, Morphology and networks of sunflower wax crystals in soybean oil organogel, Food Structure. 5 (2015) 10–20. doi:10.1016/j.foostr.2015.04.002.
[49] C.J.C. Alvarado, A.S. Galindo, L.B. Bermúdez, C.P. Berumen, C.Á. Orta, J.A.V. Garza, Cera de Candelilla y sus aplicaciones, Avances En Quimica. 8 (2012) 105–110.
[50] S. Murdan, G. Gregoriadis, A.T. Florence, Inverse toroidal vesicles: Precursors of tubules in sorbitan monostearate organogels,

International Journal of Pharmaceutics. 183 (1999) 47–49. doi:10.1016/S0378-5173(99)00042-3.
[51] H.M.A. Mohamed, K. Larsson, Effects on Phase Transitions in Tripalmitin due to the Presence of Dipalmitin, Sorbitan-Monopalmitate or Sorbitan-Tripalmitate, Lipid / Fett. 94 (1992) 338–341. doi:10.1002/lipi.19920940905.
[52] A.P.B. Ribeiro, M.H. Masuchi, E.K. Miyasaki, M.A.F. Domingues, V.L.Z. Stroppa, G.M. de Oliveira, T.G. Kieckbusch, Crystallization modifiers in lipid systems, Journal of Food Science and Technology. 52 (2015) 3925–3946. doi:10.1007/s13197-014-1587-0.
[53] M.H. Masuchi, R. Grimaldi, T.G. Kieckbusch, Effects of sorbitan monostearate and monooleate on the crystallization and consistency behaviors of cocoa butter, JAOCS, Journal of the American Oil Chemists' Society. 91 (2014) 1111–1120. doi:10.1007/s11746-014-2469-3.
[54] S. Murdan, G. Gregoriadis, A.T. Florence, Novel sorbitan monostearate organogels, Journal of Pharmaceutical Sciences. 88 (1999) 608–614. doi:10.1021/js980342r.
[55] S.S. Sagiri, U. Kasiviswanathan, G.S. Shaw, M. Singh, A. Anis, K. Pal, Effect of sorbitan monostearate concentration on the thermal, mechanical and drug release properties of oleogels, Korean Journal of Chemical Engineering. 33 (2016) 1720–1727. doi:10.1007/s11814-015-0295-4.
[56] M.A. Stahl, Estruturação de sistemas lipídicos com obtenção de gorduras zero trans com teores reduzidos de ácidos graxos saturados, UNICAMP, 2015.
[57] G.M. De Oliveira, M.A. Stahl, A.P.B. Ribeiro, R. Grimaldi, L.P. Cardoso, T.G. Kieckbusch, Development of zero trans/low sat fat systems structured with sorbitan monostearate and fully hydrogenated canola oil, European Journal of Lipid Science and Technology. 117 (2015) 1762–1771. doi:10.1002/ejlt.201400559.
[58] U.S. National Library of Medicine, Toxicology data network, SORBITAN MONOSTEARATE. (1999). https://toxnet.nlm.nih.gov/cgi-bin/sis/search2/r?dbs+hsdb:@term+@DOCNO+778.
[59] H.S. Hwang, M. Singh, E.L. Bakota, J.K. Winkler-Moser, S. Kim, S.X. Liu, Margarine from organogels of plant wax and soybean oil, JAOCS, Journal of the American Oil Chemists' Society. 90 (2013) 1705–1712. doi:10.1007/s11746-013-2315-z.
[60] K.F. Chaves, Processo simplificado de fabricação de margarinas com reduzidos teores de ácidos graxos saturados utilizando a tecnologia de estruturação de óleos, UNICAMP, 2014.
[61] E. Yilmaz, M. Ogutcu, Oleogels as spreadable fat and butter alternatives: sensory description and consumer perception, The Royal Society of Chemistry. 5 (2015) 50259–50267. doi:10.1039/C5RA06689A.
[62] D.C. Zulim Botega, A.G. Marangoni, A.K. Smith, H.D. Goff, Development of formulations and processes to incorporate wax oleogels in ice cream, Journal of Food Science. 78 (2013) 1845–1851. doi:10.1111/1750-3841.12248.
[63] A. Jang, W. Bae, H.S. Hwang, H.G. Lee, S. Lee, Evaluation of canola oil oleogels with candelilla wax as an alternative to shortening in baked goods, Food Chemistry. 187 (2015) 525–539. doi:10.1016/j.foodchem.2015.04.110.
[64] B. Mert, I. Demirkesen, Reducing saturated fat with oleogel/shortening blends in a baked product, Food Chemistry. 199 (2016) 809–816. doi:10.1016/j.foodchem.2015.12.087.
[65] H.L. Bemer, M. Limbaugh, E.D. Cramer, W.J. Harper, F. Maleky, Vegetable organogels incorporation in cream cheese products, Food Research International. 85 (2016) 67–75. doi:10.1016/j.foodres.2016.04.016.

Edible films: the package of the future?

T.G. Cerón Carrillo and **N.A. Santiesteban-López**

Benemérita Universidad Autónoma de Puebla. Avenida San Claudio y 22 sur, Jardínes de San Manuel. C.P. 72570 Puebla, Puebla, Mexico

Nowadays, there is a concern about the methods used for food preservation. One of the most important is that the food industry has been using additives to extend the shelf life of products, which at times, does not turn out to be quite safe, because of the assumption that most of them have health risk associated with cancer, asthma, cardiovascular diseases, allergies, etc. So, their use is justified for the simple fact of reducing costs because of lack of awareness of the producer. In addition, the amount of food waste has increased annually, exacerbating the situation and therefore, increasing the concern of finding the best way to preserve food without changing its physical and nutritional properties at the lowest price.

The use of edible films in the food industry has evolved. Today, the impact they generate, as a means of conservation, is great, because they are composed of polymers, with specific characteristics, of natural origin. They can be polysaccharides, animal and vegetable proteins and lipids, which, when added to food reduce the loss of moisture, also they act as a barrier for CO2 and O2 movement through the food product, and improve they mechanical, physical and nutritional properties of food. On the other hand, they serve as active vehicles for bacteriocins, probiotics, antioxidants and nutrients, each of them having specific benefits in the proper functioning of the organism, optimizing the product for human consumption. Therefore, the present review article has as main objective to highlight the importance of edible films as packages and their application as vehicles of various bioactive compounds, and discuss whether in the future, they would be one of the best sustainable alternatives for the conservation and commercialization of processed foods.

Keywords: edible films; edible coating; edible packages; active films

1. Why use edible films?

One of the main concerns of the food industry is to find suitable packages to market the food and to keep it in good condition for longer. These packaging are intended to be practical and malleable but with sufficient permeability to prevent food contamination by microorganisms, dust, gases or moisture.

Such packaging is expected to prolong the life of the product on the shelf and at the same time retaining its organoleptic properties. Due to increasing pollution and deterioration of resources worldwide in recent years, the industry has opted for organic materials and made from renewable resources to pack their products.

Edible films in the food area function as selective barriers for the transference of gases, moisture and nutrients; Are used because they greatly help to reduce the deterioration of foodstuffs caused by environmental factors. Likewise, it is sought to avoid or reduce the oxidation and loss of volatile compounds responsible for specific flavors and properties of food, therefore, the main objective of this chapter is to discuss if edible films made especially for fresh produce

2. What we know?

Nowadays, most consumers are worried about, not just what they eat and its nutritional characteristics, but also, they are worried about if the food that they eat contains some type of hazardous materials and even more, they are worried about its sustainable characteristics.

As consumers, we all want to eat fresh products, but what we don´t know is how far is the food from the place that has been picked or obtained directly. Now, the time that takes our food to get to our home and to our mouth, could be from hours to days, and this is how the “problem” begins. As soon as the fruit, vegetable, meat or any animal products are obtained, its aging starts.

But, how do we know that some products are fresh? the answers is in its flavor, its appearance and its texture. Therefore, food scientists are focused on generate technologies that maintained product’s “fresh like” characteristics for large periods of time.

People have been used some of these technologies for ages. Examples involve, increasing temperature to eliminate pathogens (pasteurization), decrease temperature to prevent bacteria and mold growth (refrigeration and freezing), adding salt or sugar also to prevent bacterial growth (salting and crystallization), etc. The issue with these technologies is that all of them affect either way sensorial quality of food products.

So, as an intent to decrease these changes, producer from China in the year 1100, and to maintain Emperor’s food fresh (especially citrus fruits) they used molten wax to cover them for long journeys [1] . Latter, this practice was extended through Europe, known as “larding”, in which they also used fats to prevent spoilage, nevertheless, this form to preserve fruits, altered its taste and texture [2]

Then, Japanese used a coat obtained in the processes of boiling soy milk (named yuba) preserving overall quality of food [3]. Later, other technologies came along, such as, smoking, cooling (in iceboxes) and the used of heat. However, coating of products was always a cheap way to maintain their safety and quality.

We could now stablish that an edible film is any primary package (in direct contact with food) used to extend food product shelf life with its quality and sensory characteristics unchanged, with the unique characteristic that could be eaten with the food that contain them. The difference between an edible film and an edible coat is that the first is made to function as a wrap or a bag to cover the product and is usually from 50 to 250 mm thick. On the other hand, edible coating is formed in the product and it could be applied by a variety of method like, brushing, spraying, or dipping.

Edible films and coatings have several applications: they could serve as a barrier against the movement of molecules such as water vapour, ethylene, carbon dioxide, odor molecules, among others. They also could be used as carrier of active compounds like nutrients, antioxidants agents, pigments, antibacterial components to add beneficial effects to the food. The other important property of the edible films is to enhance of food's sensory properties [4]

Food's characteristics determined the type of edible films that can be applied, for instance, polysaccharides and protein based films are used as to carry nutrients and additives due to their bonding capacity, but they could not be used to avoid water loss because of their hydrophilic condition. Fat based edible films tend to break easily due to their no polar solubility but, also because of this, they are very good as water barrier [5]

Protein are a well-known material for films production since they can interact with several components through their amino acid chains creating a cohesive and stable film or coat [6]

Some of the protein used are: casein, whey, gelatin, soy protein, keratin, egg albumen, wheat protein, corn zein and depending on the ingredient the preparation could change.

Gennadios et al. [7] observed that gelatin could be used as a barrier to reduce oxygen, moisture and oil transfer when applied to meat products, but also its applicability is limited because is considered as a hydrophilic material, which means that it doesn't work as a good water vapor barrier. In another research made by Guilbert [8], it was probed that zein has an excellent films formation protein and that it has better water vapor barrier characteristics in comparison with films produced with other protein, it reduces moisture loss and delay color change when applied in fresh fruit. But at the same time, the films made with this protein, need to be combined with fatty acids or with cross-linking reagents to improve its barrier properties.

Gennadios and Weller [9] worked with gluten based films, and they concluded that films made only with gluten, present low flexibility, thus the need to add plasticizer (glycerin), although, they reduce their water vapor barrier characteristics, its strength and elasticity.

In another research conducted by Zhang et al. [10] in which they worked with soy protein, they observed that the films have a high level of stiffness, tensile strength and developed films that are inexpensive and nutritional. On the other hand, casein films showed good free standing formation characteristics, but poor water vapor properties, they also transparent and flexible [11]

When films are produce with polysaccharide gums and naturally depending on the material used, they present certain and specific characteristics, such as plasticity, tensile strength, clarity and solubility due to the hydrogen bonding [12] Some of the gums used in film forming process are methylcellulose, alginate, carboxymethylcellulose, carrageenan, gellan, locust bean, agar, among others.

Nieto [12] concluded that films made with agar are clear, strong, insoluble to water and form stable networks that could be peel off improving its manageability however, they are brittle and with reduce elasticity properties.

Murray [13] showed that films with methylcellulose could be peel off and that they have high levels of tensile strength but it is a unique material since the process must have such heating controls for the gel phase to hold. Similarly, Konjac gum is a hydrocolloid obtained from the common Chinese plant known as *Amorphophallus spp*., according to Takigami [14], this gum produces strong films, due to its possibility to increase viscosity at low concentration, however, this property is could be too the downside for used Konjac gum, making solutions too viscous.

Yuen [15] worked with pullulan, a polysaccharide obtained from fungus *Aureobasidium pullulans*, and observed that the compounds formed a clear, strong and like synthetic polymers, their used in food products is due to its low permeability to oxygen and its high solubility in water.

According to Donati et al. [16] alginate associated with calcium form heat stable gels which also form films with increased tensile strength. Nevertheless, their negative charges, could decreased tensile strength and increased water solubility. On the other hand, Carrageenan, which is also a polysaccharide extracted from a seaweed, possess three different conformation; kappa, iota and lambda each with different characteristics: kappa formed the strongest films of all [17]. Similarly, Gellan gum (obtained from the fermentation process of *Sphyngomonas elodea*, can be classified in two; high acyl (with high flexibility, soft, and transparent) and low acyl (hard and non-elastic gels). Gellan gum form weak films, with low tensile and puncture strength but they are also clear and insoluble in cold water [18].

Pectin has shown to require certain specification to fully hydrate hence, to create gel and films, such as sugar content (from 20 to 55%), pH (as low as 3.5) and temperature of process (from 50 to 85ªC). Pectin films are weaker than alginate films [19]. Chitosan, the principal material of the exoskeleton of crustaceans, formed films strong enough to made plastic wraps for fresh produce, they have high tensile and puncture strength [20].

Fats also are used as ingredient in film production because they are very effective barriers against moisture, meaning, water cannot enter or get out of the food [21] Some of this components are: waxes, margarine, lacs, shortening, resins, essential oils, fatty acids, among others.

Although most of the ingredients by themselves could be used to elaborate edible films, there's some problems or weak points in each of them (Table 1).

Table 1 Advantages and disadvantages of some of the most used ingredients in edible films.

Material	Advantages	Disadvantages
Protein		
Casein [22]	Good barrier to oxygen	Tend to shrink, brittle
Whey [23]	Water insoluble, flavorless, flexible, high clarity,	Need a process of crosslinking, texture depends on the aminoacid composition
Gelatin [24]	Odorless, cheap, transparent, tasteless	Water soluble, hence limited used in food industry
Gluten [3]	Good oxygen barrier, good tensile strength and elongation break	Poor water vapor barrier, opaque films,
Soy [10]	Inexpensive, environmental friendly, nutritional	Poor moisture barrier, brittle and stiff.
Zein [25]	Flexible, smooth, cheap, good oxygen barriers	water solubility, poor mechanical properties, poor heat sealability
Polysaccharide		
Alginate [26]	Strong films, low permeable to oxygen, tasteless, odorless, glossy	High water vapor permeability
Carrageenan [27]	Solid films, low permeability, high tensile strength	Opaque, low values of elongation at break, poor heat sealability
Gellan [28, 29]	Thermal stability, resistant to acid, clear films, poor solubility in water, clear	High water sorption capacity, brittle and hard
Agar [30]	Good mechanical properties, transparent, flexible, clear	Only soluble in hot water, high swelling capacity
Carboximethylcellulose [31]	Smooth, low water vapor permeability.	Low elongation at break point, low tensile strength
Pullulan [32]	High gas barrier, glossy, water soluble, odorless, tasteless.	Could become sticky, easily soluble at high levels of relative humidity

3. Novel composite films

Now, since any material alone does not have all the properties that consumers want; low water vapor permeability for fresh produce, high flexibility, high tensile and puncture strength and biodegradability, researcher have been studied the combination of two or more materials, composite films. The material, concentration and application mode depends entirely upon the type of food.

Many are the research that have been conducted in composite films; according to Galus and Lenart [33] a composite film made with alginate and pectin shows higher values of tensile strength and elongation at break rather than the ingredients individually. Also, they proved to generate clear and homogeneous films that also had lower water vapor sorption in comparison with alginate only films.

In another work conducted by Otoni et al. [34], they evaluated the properties of a soy protein-oil film and they found that the water barrier properties increased when oil is added to the soy protein solution, in addition, total pore volume decrease. Alvarado-Gonzalez et al. [29] work with a blend of *Aloe vera* and gellan gum to improve their physical and chemical properties, and they found that the combination shows higher levels of transparency, water sorption capacity and water vapor permeability were enhanced. In the same way, Eghbal et al. [35] probed that when sodium caseinate and pectin, tensile strength and elongation at break improved and water sorption decreased.

However, not all blends improve films properties. One example is the research conducted by Jridi et al. [36], they observed that when combined gelatin and chitosan, the composite films shows no significant different in transparent and tensile strength than the observe in chitosan films.

Other researches have been investigated the blend of more than two materials; Tong et al. [37], studied the effect of combine Pullulan, alginate and carboxymethylcellulose in edible films properties. The resultant films had better mechanical, barrier and water solubility properties. Similar research made by Taqi et al. [38] probed that when apple pectin, cassava starch, essential oils and oleic acid are combined, its chemical and physical properties were changed; tensile strength decreased and the elongation at break point increased.

Composite edible films in food industry have been used to maintain fresh fruits microbial, physical and chemical properties. Some other works are show in the list below (Table 2)

Table 2 Recent research on composite edible films.

Ingredients	Changes observed	Reference
Cutin/pectin	Lower water uptake, lower solibility	[39]
Kappa carrageenan/sorbitol/glycerol	Higher tensile strength, higher elongation at break, increased moisture content, water solubility and water vapor solubility	[27]
Gelatin/curcumin	Film change color with pH	[40]
Aloe vera/plpantain flour	Smoother, clear, rigid and plastic films	[41]
Whey protein/pectin/transaglutaminase	Prevent weigh loss of fresh cut produce, prevent microbial growth	[42]
Mesquite seed gum/palm fruit oil	Decrease water solubility, decreased water vapor permeability, improve tensile strength	[43]
Moringa leaf extract/chitosan/carboxymethylcellulose	Lower mass loss, lower respiration rate	[44]
Chitosan/gum Arabic	Lower mass loss, lower respiration rate and ethylene production	[45]
Pomegranate peel pectin/montmorillonite	Improve tensile strength, water vapor permeability decrease	[46]
Shellac/gelatin	Decrease weight loss, maintain post harvest quality of banana for more than 30 days	[47]
Gelatin/defatted soy protein	Increase color properties, tensile strength, water permeability, water solubility	[48]
Water chestnut starch/chitosan/glycerol monolaureate/ nisin/pine needle essential oil	Lower water absorption expansion, tensile strength elongation and puncture strength	[49]
Rice starch/L-carrageenan	Improve tensile strength and elongation at break, better solubility enhance water vapor permeability	[50]
Beeswax/hydroxypropyl methylcellulose	Reduce oxygen barrier, reduce mechanical resistance, improve moisture barrier, reduce weight loss	[51]
Nanoclay/quince seed mucilage	Improve tensile strength, increase elongation, improve gas barriers	[52]

4. Active films

So far, information about edible films and coating with improve physical and chemical properties have been well studied, however, recently, researches has taken notice in the importance and application of bioactive compounds added to edible films. The purpose is to enhance antioxidant, antimicrobial or even nutritional characteristic of the food that contain them, hence, its name “active films”.

One of the most studied bioactive component is essential oil to prevent microbial growing. Hashemi and Khanaghah [53] added oregano essential oil to basil seed gum based edible films and observed antibacterial activity with 2-6% content of oregano essential oil against *Escherichia coli, Salmonella Typhimurium, Pseudomona aureginosa, Staphylococcus aureus* and *Bacillus cereus*. Another research conducted by Kazerani et al. [54] they probed antibacterial effects against *Staphylococcus aureus* and *Escherichia coli* when *Zataria multiflora* essential oil was added to cress seed gum based edible films, also, they found that the addition of essential oil caused a change in glass temperature transition of the film.

Not only antibacterial effect has been studied, also work about antifungal effect of some bioactive component has been made, Bahram et al., [55] evaluated the effect of cinnamon essential oil against *Candida albicans*, when applied to whye concentrate edible films and the concluded that the film exhibited good inhibitory effect against fungi. Also, Gniewozs et al., [56] showed that with 0.12% of added Caraway essential oil to Pullulan based edible films, *Aspergillus niger* was inhibited along with the population of *Salmonella enteriditis, Staphylococcus aureus, Saccharomyces cerevisae* in fresh baby carrots.

The impact of probiotics in human health have been extensively studied, due to the increased interest of consumer in wellness therefore biopolymeric matrices have been added with probiotics as an alternative to enhance food safety and health. Soukoulis et al. [57] estudied the viability of *Lactobacillus rhamnosus* incorporated in edible films, and concluded that two blends; high viscosity alginate/whey protein and k-carrageenan/locust beam gum/whey protein had the best mechanical and barrier properties. In the same way, pullulan and pullulan /potato starch are the best carriers for *Lactobacillus plantarun, Lactobacillus reuteri* and *Lactobacillus acidophilus* with 80% of viable cells and after 2 months of storage [58], Lopez de Lacey et al., [59] also probed that biopolymers are excellent carriers for probiotics, they incorporated *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in gelatin and observed that probiotic decreased in less that 2 log cycles.

According to Percival [60] an antioxidant compound is the one that “are capable of stabilizing and deactivating free radicals before they attack cells. Antioxidants are absolutely critical for maintaining optimal cellular and systemic health and well-being”. Because of the edible films are good carriers for these compounds, giving a functional property

to the food matrix. Suppakul et al. [61] evaluated the radical scavenging and ferric reducing antioxidant power and observed that when combined Indian Goosberry extract to a composite Indian Goosberry puree/methylcellulose based films, it showed the higher antioxidant activity during 90-day storage. Antioxidant compounds are found mainly in plants, such as sage and laurel, which when incorporate to whey based edible films, contribute to the oxidative stability of frozen meatballs [62].

Finally, it could be also incorporate other type of nutrients such as vitamins and minerals; this is the case of the research made by Bilbao-Sainz et al. [63] were they added vitamin D2 to an edible mushroom chitosan based edible films. Another vitamin that was incorporated to edible films is vitamin E or tocopherol and when added to chitosan edible films, decreased film's solubility and increased significantly its drying rate [64].

5. A green option: Edible packages

Other used that has been given, and recently is getting importance from the consumer, is the "green" side of apply them as a wrapping or as a package that would reduce plastic waste. Plastic take thousands of years to break down, meanwhile it would become a contamination problem affecting all types of living beings. So, to counteract the problem, experts proposed two options; to recycle plastic or to gradually change to biodegradable materials. However, recycling involves transporting and sorting costs leaving biodegradable materials in first place to a cost-effective solution. Still, biodegradable material should be created from raw ingredients that would be disposable in other product manufacture.

So, the material used should be safe and nutritious for the people not to dispose any wrapping or packaging material when eating food.

A great example of this is protein based film which have been proven to be biodegradable and compostable source giving nitrogen to the soil to fertilized it [65] being casein a waste product in dairy process. Semolina loaded with nano kaolin have been probed to also make a suitable material for biodegradable edible package [66].

Recently, certain companies launched products with edible packages; Saltwater Brewery ®, create from beer by-products and edible ring used in their beer's six packs, these rings feed marine animals instead of killing them [67].

In the same way, Rodrigo García González a postgraduate student of the Royal College of Art innovate with egg yolk a package that could allegedly, replace water bottles. The design consists in a sphere like package containing water (with the appearance of a jellyfish), that could be thrown away or could be eaten [68]

Is worth mention that not only small producers are interested in this type of packages; for instance, KFC®, introduced a tortilla based bowl "Rice bowlz" as an Indian product, the good news is that this product was well adopted by consumers, this was a initiative that begins when the government of Bangalore banned plastic [69]

Wikicells® is another innovative way to package foodstuff, the brand was invented by Dr. David Edwards whose ideas came from the model of "nature to wrap food". This type of wrapping involves several plastic-like ingredients such as cookie dough, cocoa powder and chia to contain from frozen yogurt to gaspachio [70].

6. Conclusions

After all the facts that were mentioned above, we could conclude that edible packages are now the ultimate way to present processing food. It can be used several ingredients; polysaccharides, proteins and lipids or fats.

Researches have turn around their eyes to this opportunity area of create a new, innovative way to package food, incorporating ingredients that the food does not have such as antioxidants, probiotics, vitamins flavor among others. Many times, ingredients by themselves don't have the properties that the science looking for to contain different types of food, so, composite films (combinations of two or more components) are the best way, to improve material's characteristics such as tensile strength, odor, taste, water sorption, water permeability, flexibility, etc.

Now, small and big companies are seeking out the best, low cost, greener way to sell their products being in recent years when investigations of this type have pay out.

So, we can say that eventually, and hopefully, edible and functional packaging would be the only way in which food would be commercialized to; improve health, lower contamination damage (to us and to the environment), increased product's shelf life and finally increase cost-benefit ratio of foodstuff.

Acknowledgments We would like to thank to the Dirección de Superación Académica (DSA) of the Mexican goverment and the Benemérita Universidad Autónoma de Puebla for the grant conceded to PhD Teresa Gladys Cerón Carrillo.

References

[1] Hardenburg RE. Wax related coatings for horticultural products. A bibliography. Agricultural Research Service Bulletin. 1967; United States Department of Agriculture, Washington, DC

[2] Contreras-Medellin R, Labuza TP. Prediction of moisture protection requirements for foods. Cereal Food World.1981; 26(7): 335–340

[3] Gennadios A, Weller CL, Testin RF. Modification of physicaand barrier properties of edible wheat gluten-based fils. Cereal Chemistry. 1993; 70(4):426-429
[4] Pascall MA, Lin SL. The application of edible polymeric films and coatings in the food industry. Journal of Food Processing and Technology. 2013; 4(2): 100e116
[5] Hernandez-Izquierdo VM, Krochta JM. Thermoplastic processing of proteins for lm formation a review. J Food Science. 2008; 73: R30-R39.
[6] Barone JR, Schmidt WF. Nonfood applications of proteinaceous renewable materials. Journal of Chemical Education. 2006; 83(7): 1003–1009
[7] Gennadios A, McHugh TH, Weller CL, Krochta JM. . Edible coating and films based on protein. In: Krochta J M, Balwin EA, Niperos Carriedo M O, editors. Edible Coatings and Films to Improve Food Quality. Lancaster, Technomic Publishing; 1994. p. 201-277
[8] Guilbert S. Technology and application of edible protective films. In: Matholouthi M. editor. Food packaging and preservation, Theory and Practice. London: Elsevier Applied Science Publishing; 1986. pp. 371-394.
[9] Gennadios A, Weller CL. Edible films and coating from wheat and corn proteins. Food Technology, 1990; 44(10): 63-69.
[10] Zhang, J., P. Mungara, J. JaneMechanical and thermal properties of extruded soy protein sheets. Polymer. 2001; 42: 2569-257
[11] Avena-Bustillos, R. J.; Cisneros-Zevallos, L. A.; Krochta, J. M. & Saltveit, M. E. Optimization of edible coatings on minimally processed carrots using response surface methodology. Transactions of the ASAE. 1993; 36(3): 801-805.
[12] Nieto MB. 2009. Structure and function of polyssacharide gum based edible films and coating. In Embuscado ME, Huber KC. Editors. Edible films and coatings for food applications. New York, EUA. Springer. 2009. p 57-112.
[13] Murray JCF. Cellulosics. In: Phillips GO, Williams PA. editors. Handbook of Hydrocolloids. Cambridge: Woodhead Publishing Ltd; 2000. p 219–229
[14] Takigami S. Konjac Mannan. In: Phillips GO, Williams PA. editors. Handbook of Hydrocolloids. Boca Raton: CRC/Woodhead Publishing; 2000. p 413–424
[15] Yuen S. Pullulan and its applications. Process Biochemistry. 1974; 9:7–9
[16] Donati I, Holtan S, Mørch YA, Borgogna M, Dentini M, Skjåk-Bræk. New hypothesis on the role of alternating sequences in calcium-alginate gels. Biomacromolecules. 2005; 6:1031–1040
[17] Falshaw R, Bixler HJ, Johndro K. Structure and performance of commercial kappa-2 carrageenan extracts I. Structure analysis. Food Hydrocolloids. 2001; 15:441–452
[18] Sworn G. Gellan gums. In:. Phillips GO, Williams PA, editors. Handbook of Hydrocolloids. Boca Raton: CRC/Woodhead Publishing; 2000. pp 117–35
[19] May CD. Pectins. In: Phillips GO and Williams, PA, editors. Handbook of Hydrocolloids. Cambridge: Woodhead Publishing Ltd; 2000. pp. 219–229
[20] Srinivasa P, Baskaran R, Ramesh M, Harish Prashanth K, Tharanathan R. Storage studies of mango packed using biodegradable chitosan film. European Food Research and Technology. 2002; 215(6):504–508
[21] Morillon V, Debeaufort F, Blond G, Capelle M, Voilley A. Factors affecting the moisture permeability of lipid-based edible films: a review. Critical Reviews in Food Science and Nutrition. 2002; 42(1):67–89
[22] Mauer LJ; Smith DE, Labuza TP. Water vapor permeability, mechanical, and structural properties of edible beta-casein films. International Dairy Journal. 2000;10, 353–358.
[23] Pérez-Gago MB, Nadaud P, Krochta JM. Water vapor permeability, solubility, and tensile properties of heat-denatured versus native whey protein films. Food Technology. 1999; 64(6):1034-1037.
[24] Ramos M, Valdés A, Beltrán A, Garrigós MC. Gelatin-based films and coatings for food packaging applications. Coatings. 2016; 6:41: 1-20
[25] Bradenburg AH, Weller CL, Testin RF. Edible films and coatings from soy protein. Journal of Food Science, 1993; 58(5): 1086-1089.
[26] Pranoto Y, Salokhe VM, Rakshit SK. Physical and antibacterial properties of alginate-based edible films incorporated with garlic oil. Food Research International. 2005; 38:267-272
[27] Farhan A, Hani NM. Characterization of edible packaging film based on semi-refined kappa-carrageenan plasticized with glycerol and sorbitol. Food Hydrocolloids. 2017; 64:48-58
[28] Jampen S, Britt IJ, Tung MA. Gellan polymer solution properties: Dilute and concentrated regimes. Food Research International. 2000; 33: 579–586
[29] Alvarado-Gomzalez JS, Chanona-Pérez JJ, Welti-Chanes JS, Calderón-Domínguez G, Arzate-Vázquez I, Pacheco-Alcalá SU, Garibay-Febles V, Gutierrez-López GF. Optical, microestructural, functional and mechanical properties of Aloe vera gel/Gellan gum edible films. Revista Mexicana de Ingeniería Química. 2012; 11(2):193-210.
[30] Phan The D, Debeaufort F, Voilley A, Luu D. Biopolymer interactions affect the functional properties of edible films based on agar, cassava starch and arabinoxalyn blends. Journal of Food Engineering. 2009; 90(4):548-558
[31] Martelli SM, Motta C, Caon T, Alberton J, Belletini IC, Do Prado ACP, Barreto PLM, Soldi V. Edible carboxymethyl cellulose films containing natural antioxidant and surfactants: a-tocopherol stability, in vitro release and films properties. LWT Food Science and Technology. 2017; 77:21-29
[32] Rhim JW. Characteristics of Pullulan based-edible films. Food Science Biotechnology. 2003; 12(2):161-165
[33] Galus S, Lenart A. Development and characterization of composite edible films based on sodium alginate and pectin. Journal of Food Engineering. 2013; 115(4):459-465
[34] Otoni CG, Avena-Bustillos RJ, Olsen CW, Bilbao-Sáinz C, McHugh TH. Mechanical and water barrier properties of isolated soy protein composite edible films as affected by carvacrol and cinnamaldehyde micro and nanoemulsions. Food Hydrocolloids. 2016; 57, 72–79.
[35] Eghbal N, Yarmand MS, Mousavi M, Degraeve P, Oulahal N, Gharsallaoui A. Complex coacervation for the development of composute edible films based on LM pectin and sodium caseinate. Carbohydrate Polymers. 2016; 151: 947-956

[36] Jridi M, Hajii S, Ayed HB, Lassoued I, Mbarek A, Kammoun M, Souissi N, Nasri M. Physical, structural, antioxidant and antimicrobial properties of gelatin-chitosan composite edible films. Macromolecules. 2014; 67:373-379
[37] Tong Q, Xiao Q, Lim L. Preparation and properties of pullulan-alginate-carboxymethylcellulose blend films. Food Research International. 2008; 41:1007-1014
[38] Taqi A, Mutihac L, Stamatin I. Physical and barrier properties of apple pectin/cassava starch composite films incorporating lauris nobilis L. oil and oleic acid. Journal of Food Processing and Preservation. 2014; 38:1982-1993.
[39] Manrich A, Moreira FKV, Otoni CG, Lorevice MV, Martinis M, Mattoso LHC. Hydrophobic edible films made up of tomato cutin and pectin. Carbohydrate polymers. 2017; 164:83-91
[40] Musso YS, Salgado PS, Mauri AN. Smart edible films base don gelatin and curcumin. Food hydrocolloids. 2017; 66:8-15.
[41] Gutierrez TJ, González G. Effect of cross-linking with Aloe vera gel on surface and physicochemical properties of edible films made from plantain flour. Food Biophysics. 2017;12(1):11-22
[42] Marquez GS, Di Perro P, Marinello L, Esposito M, Giosafatto CVL, Porta R. Fresh cut fruit and vegetable coatings by transaglutaminase-crosslinked whey protein/pectine edible films. LWT Foo Science and Technology. 2017; 75:124-130
[43] Rodrigues DC, Cunha AP, Brito ES, Azeredo HMC, Gallao MI. Mesquite seed gum and palm fruit oil emulsion edible films: influence of oil content and sonication. Food hydrocolloids. 2016; 56:227-235
[44] Tesfay SZ, Magwaza LS. Evaluating the efficacy of moring leaf extract, chitosan and carboxymethylcellulose as edible coatings for enhancing quality and extending postharvest life of avocado (Persea amaericana Mill.)fruit. Food packaging and shelf life. 2017; 11:40-48
[45] Khaliq G, Mohamed MTM, Ding P, Ghazali HM, Ali A. Storage behavior and quality responses of mango (*Mangifera indica L.*) fruit treated with chitosan and gum arabic coatings during cold storage condictions. International Food Research Journal. 2016; 23:S141-S148
[46] Oliveira TIS, Zea-Redondo L, Moates GK, Wellner N, Cross K, Waldron KW, Azeredo HMC. Pomegranate peel pectin films as affected by montmorillonite. Food Chemistry. 2016; 198:107-112
[47] Soradech S, Nunthanid J, Limmatvapirat S, Luangtana-anan M. Utilization of shellac and gelatin composite film for coating to extend the shelf life of banana. Food Control. 2017; 73:1310-1317.
[48] Tulamandi S, Rangarajan V, Rizvi SSH, Sighal RS, Chattopadhyay SK, Saha NC. A biodegradable and edible packaging film based on papaya puree, gelatin, and defatted soy protein. Food Packaging and Shelf Life. 2016; 10:60-71
[49] Mei J, Yuan Y, Guo Q, Wu Y, Li Y, Yu H. Charaterization and antimcirobial properties of wáter chestnut starch-chitosam edible films. International Journal of Biological Macromolecules. 2013; 61:169-178
[50] Thakur R, Saberi B, Pristijono P, Golding J, Stathopoulos C, Scarlett C, Bowyer M, Vuong Q. Characterization of rice starch l-carrageenan biodegradable edible films. Effect of stearic acid on the films properties. International Journal of Biological Macromolecules. 2016; 93(A):952-960
[51] Navarro-Tarazaga ML, Massa A, Pérez-Gago MB. Effect of beeswax content on hydroxypropyl methylcellulose-based edible film properties and postharvest quality of coated plums (Cv. Angeleno). LWT Food Science and Technology. 2011; 44(10):2328-2334.
[52] Shekarabi AS, Oromiehie AR, Vaziri A, Ardjmand M, Safekordi AA. Investigation of the effect of nanoclay on the properties of quince seed mucilage edible films. Food Science and Nutrition. 2014; 2(6):821-827
[53] Hashemi SMB, Khaneghah AM. Characterization of novel basil-seed gum active edible films and coating containing oregano essential oil. Progress in Organic Coatings. 2017; 110:35-41
[54] Kazerani H, Fahimdanesh M, Jouki M, Tayefi F, Tayyari F. Physical, mechanical, thermal and antimicrobial properties of active edible films made from cress seed gum incorporated with Zataria essential oil. Carphatian Journal of Food Science and Technology. 2016; 8(4):83-90.
[55] Bahram S, Rezaei M, Soltani M, Kamali A, Ojagh SM, Abdollahi M. Whey protein concentrate edible film activated with cinnamon essential oil. Journal of food Processing and Preservation. 2014; 38:12511258
[56] Gniewozs M, Krasniewska K, Woreta M, Kosakowska O. Animicrobial activity of a pullulan –caraway essential oil coating on reduction of food microorganisms and quality in fresh baby carrot. Journal of Food Science. 2013; 78(8):M1242-8
[57] Soukoulis C, Behboudi-Jobbehdar S, Macnaughtan W, Parmenter C, Fisk ID. Stability of *Lactobacillus rhamnosus* GG incorporated in edible films: impact of anionic biopolymers and whey protein concentrate. Food hydrocolloids. 2017; 70: 345-355
[58] Kanmani P, Lim ST. Development and characterization of novel probiotic-residing pullulan/ starch edible films. Food chemistry. 2013; 141(2):1041-1049.
[59] Lopez de Lacey AM, Lopez-Caballero ME, Gómez-Estaca J, Gómez Guillén MC, Montero P. Functionality of Lactobacillus acidophilus and Bifidobacterium bifidum incorporated to edible coatings and films. Innovative Food Science and Emerging Technologies. 2012; 16:277-282.
[60] Percival M. 1998. Antioxidants. Clinical Nutrition Insights. Available in https://acudoc.com/Antioxidants.PDF.
[61] Suppakul P, Boonlert R, Buaphet W, Sonkaew P, Luckanatinvong V. Efficacy of superior antioxidant Indian gooseberry extract-incorporated edible Indian gooseberry puree/methylcellulose composite films on enhancing the shelf life of roasted cashew nut. Food Control. 2016; 69:51-60.
[62] Akcan T, Estevez M, Serdaroglu M. Antioxidant protection of cooked meatballs during frozen storage by whey protein edible films with phytochemicals form *Laurus nobilis L.* and *Salvia officinalis*. LWT-Food Science and Technology. 2017; 77:323-331
[63] Bilbao-Sainz C, Chiou B-S, Wolliams T, Wood D, Du W-X, Sedej I, Ban Z, Rodov V, Poverenov E, Vinokur Y, McHugh T. Vitamin D-fortified chitosan films from mushroom waste. Carbohydrate Polymer. 2017; 167:97-104.
[64] Lin SL, Pascall MA. Incorporation of vitamin E into chitosan and its effect on the film forming solution (viscosity and drying rate) and the solubility and thermal properties of the dried film. Food hydrocolloids. 2014; 35:78-84

[65] Aimutis WR. Bioactive properties of milk proteins with particular focus on carcinogenesis. Journal of Nutrition. 2004; 134849:989S-95S

[66] Jafarzadeh S, Alias AK, Ariffin F, Mahmud S, Najafi A, Sheibani S. Characterization of a new biodegradable edible film from semolina loaded with nano kaolin. International Food Research international. 2017; 24(1):304-309.

[67] Wanshel E. Edible rings on six packs feed marine life if they end up in the ocean [internet]. Huffpost. 2016 [cited 2017 june 13]. Available from: http://www.huffingtonpost.com.mx/entry/saltwater-brewery-edible-six-pack-rings-beer-plastic-marine-life_us_573b796ce4b0ef86171c5fe4

[68] Gosden E. Drunk your water? Now eat the bottle it came in. [internet] The telegraph. 2014 [cited 2017 june 16]. Available from: http://www.telegraph.co.uk/news/newstopics/howaboutthat/11095793/Drunk-your-water-Now-eat-the-bottle-it-came-in.html

[69] Boult A. KFC introduces edible bowls to replace plastic packaging [internet]. The Telegraph. 2016 [cited 2017 june 1st] Available from http://www.telegraph.co.uk/food-and-drink/news/kfc-introduces-edible-bowls-to-replace-plastic-packaging/

[70] Stevens J. Packaging you can eat: food "skins" that mimic nature could slash plastic waste [internet]. Daily mail.com. 2012 [cited 2017 may 23]. Available from: http://www.dailymail.co.uk/news/article-2160092/Packaging-eat-Food-skins-mimic-nature-slash-plastic-waste.html?ICO=most_read_module

Factors that influence color degradation in extra virgin olive oils

A. Mondragón-Valero, **D. Salazar Hernández**, **I. Rodríguez Guillamón**, **G. Hernández Hernández** and **I. López-Cortés**

Departamento Producción Vegetal. Universitat Politènica de València Camino de Vera s/n, 46022 Valencia, Spain

Nowadays olive oil is considered a global consumption product recognized by the numerous benefits it confers to human health. Its quality is regulated by the International Olive Council that establishes parameters concerning free acidity, peroxide index and organoleptic characteristics based on sensations perceived by the senses. Color is one of the most immediate organoleptic properties of olive oils determinant in consumer's choice. As olive oil is a natural product, pigments are affected and partially destroyed by the oxidative and degradation processes that the oils suffer over time during the conservation prior to consumption. This alteration of is therefore perceived today as one of the most important changes affecting the organoleptic quality of the product. The objective of this chapter is to contrast the color changes experienced by different virgin olive oils subjected to standard storage conditions and evaluate the relationship between the color alteration and the alteration of its chemical composition.

Keywords: Extra Virgin Olive Oil; Storage; Color degradation; Chemical Composition

1. Intoduction

Extra virgin olive oil (EVOO) is an important component of the praised Mediterranean diet, which is attracting increasingly the interest of scientists due to the health benefits it can provide [1]. This is due to its complex chemical composition which comprises various antioxidant substances that can be both nutritional and therapeutic. Polyphenols, sterols and fatty acids are the most influential constituents in terms of health benefits that can be found in considerable quantities in olive oil and that play a major role in human metabolism being simultaneously influent in factors such as stability, flavour and color of the oil [2].

Olive oil currently represents a small share of the whole vegetable oil market but its use is gaining ground, increasing worldwide, thanks not only to its potential health benefits but also to its unique sensory and nutritive qualities, since it is the only oil that is consumed by direct obtaining. Consumers are increasingly demanding least-processed high quality foods, and requiring quality to be maintained during a long period between purchase and consumption. Food must not only remain safe but also minimise the unwanted changes produced during the storage in the sensory quality, so it is important to evaluate the variations in a natural product as EVOO is.

2. Quality criteria in virgin olive oils

2.1 Chemical analysis

According to the International Olive Council (IOC) [3] virgin olive oils are the oils obtained from the fruit of the olive tree (*Olea europaea* L.) solely by mechanical conditions, paying particular attention to the thermal conditions, that do not lead to alterations in the oil, and which have not undergone any treatment other than washing, decantation, centrifugation and filtration. Olive oils fit for consumption as they are include:

Extra virgin olive oil: virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 0.8 grams per 100 grams, and the other characteristics of which correspond to those fixed for this category in the IOC standard.

Virgin olive oil (VOO): virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 2 grams per 100 grams and the other characteristics of which correspond to those fixed for this category in the IOC standard.

Olive oil: olive oil which has a free acidity, expressed as oleic acid, of not more than 3.3 grams per 100 grams and the other characteristics of which correspond to those fixed for this category in the IOC standard. This designation may only be sold direct to the consumer if permitted in the country of retail sale. If not permitted, the designation of this product has to comply with the legal provisions of the country concerned.

The EU regulation [4] establishes upper limit values for different oxidation indexes which are shown on Table 1.

Table 1 Particular characteristics of olive oils according to ICO trade standard 2015 [3] and Commission Regulation 1348/2013 [4].

	European Commission Regulation /International Olive Council trade standards		
Parameter	**Extra virgin olive oil**	**Virgin olive oil**	**Olive oil**
Free acidity (as oleic acid)	≤ 0.8 g % w/w	≤ 2.0 g % w/w	≤ 3.3 g % w/w
Peroxide value	≤ 20 meq O_2/kg oil	≤ 20 meq O_2/kg oil	≤ 20 meq O_2/kg oil
K_{232}	≤ 2.50	≤ 2.60	-
K_{270}	≤ 0.22	≤ 0.25	$\leq 0.30^{+i}$
ΔK	≤ 0.01	≤ 0.01	≤ 0.01

The regulation also establishes other chemical parameters related to the quality criteria of the EVOO such as the content of volatile matter or impurities and the content of phenols not detailed in this chapter.

2.2 Sensory analysis: the relevance of color

Color and appearance constitute the first contact we have with food determining our preferences and influencing our choices. Color is one of the first sensory attributes of food valued by consumers, especially in EVOO, and it can be considered a quality parameter that highly influences its acceptance and preference [5].

EVOO is obtained by simple pressing of olive fruit (Olea *Europaea*), as a natural product, it has its own color ranking from dark green to pale yellow depending on the olive variety [6, 7], the agricultural practices such as irrigation [8], the maturation index on ripeness [9] or the oil extraction process [10]. Olive fruit color suffers changes as it grows, ripens and matures moving from deep green to yellow- green and then to purple and black. The green color of the tissue is provoked by chlorophyllic and carotenoid pigments, and their concentration decreases progressively during ripening that gives way to the synthesis of anthocyanins which first appears as spots on the skin, covering later more and more of the fruit surface dyeing it in purple until full maturation, where it becomes black. Anthocyanin synthesis produces finally the pigmentation of the whole fruit invading the interior of the pulp. Only chlorophylls and carotenoids, which are fat soluble, are transferred to the EVOO, giving its highly valued natural color [15].

The extraction process of olive oil entails, to a lesser or greater extent according to the extraction conditions, a loss of pigments, which in terms of total pigment content, affects the chlorophyll pigments more than the carotenoids. Although chlorophylls and carotenoids in food are mainly valued for its chromatic function, recent research has demonstrated that these compounds can be considered as quality indicators for the end product. The pigments detected in EVOO can be divided in the ones coming from the fresh fruit as chlorophylls a and b, lutein, β-carotene, violaxanthin, neoxanthin, antheraxanthin, and β-cryptoxanthin, and those formed during the extraction process, pheophytins a and b, luteoxanthin, auroxanthin, neochrome, and mutatoxanthin. The formation of these pigments is due to the fact that during the milling process a series of acids is released from the tissues of the fruit, which in the malaxation and centrifugation period favors the pheophytinization of chlorophylls and the isomerization of the carotenoids [16].

Many factors, both agronomic and technological can affect the EVOO pigment profile to the point that color has been proposed by some authors as a characterizing factor used as quality index related to olive variety and oil extraction method [16, 17].

The objective measurement of color is of great importance for food producers due to the relationships between such attribute and the acceptability of food by consumers [14]. Despite its importance as a sensitive attribute, no objective standardized method for measuring EVOO color has yet been established. The most accepted system in worldwide industries seems to be the CIELAB colorimetric system, its application to EVOO samples provides better results than those obtained by visual methods [15].

3. Factors that influence the alteration of extra virgin olive oil quality during storage

The assurance of stability and quality of food products is therefore a matter of great importance for industrial producers. EVOO has generally a relatively long shelf-life, regulation considers periods between 12 to 18 months as the maximum storage period from bottling to consumption during which only minor changes of sensory characteristics occurs [16]. EVOO‘s nutritive properties, taste, aroma and color distinguish it from other edible vegetable oils, hence, if the worldwide prestige of EVOO is to be sustained, its quality needs to be maintained and assured throughout its commercial shelf life. It is a matter of great concern for the olive oil industry to preserve the positive attributes of oil during the time elapsing from production to bottling, and up to purchasing and consumption.

Several accelerated oxidative stability tests have been developed for fats and oils in order to obtain analytical results in a short period of time. Although these methods are useful for determining the relative oxidative stability of products,

the main drawback of the accelerated assays is that the autoxidation process takes place under drastic conditions, quite unlike those typically occurring under normal storage conditions. In fact, there is generally a lack of correlation between the VOO stability measured by means of accelerated tests and under normal storage conditions or shelf life [17].

During storage, the quality of EVOO may deteriorate because it can undergo both biological and chemical processes due to the hydrolysis of triglycerides caused by enzymes or/ and a chemical oxidation of fatty acids, promoted by the presence of oxygen and free radicals which is the main cause of EVOO degradation, resulting in the development of unpleasant odors, flavors, and on the long term a loss of nutritional quality [18] that could reduce its commercial value.

3.1 Hydrolysis of triglycerides

Hydrolysis usually produces an alteration of oil when enzymes and water are present. Under these conditions, the aqueous phase, consisting of a small quantity (approximately 0.5 %) of vegetation water, contains enzymes and, in particular, lipase, which is able to hydrolyse triglycerides to release free fatty acids and, as a consequence, increases the acidity of the oil. That reaction is promoted by storage temperatures higher than 18–20 °C [11]. If, in mill, olive oil is not separated from the sediment as quickly as possible the small drops of the emulsified water slowly settle on the bottom of the container forming a layer of sediment that contains sugars, proteins and enzymes which can ferment producing mostly short-chain fatty acids that provoke the organoleptic defect of muddy sediment or putrid.

3.2 Oxidative rancidity

Oxidative rancidity development has been recognised as the predominant cause of oil deterioration during storage [16]. Autoxidation is reaction between unsaturated fatty acids, regardless of whether they are in their free state or esterified as a triglyceride molecule, and oxygen.

The main compositional factors of olive oils that determine their susceptibility to oxidation are the fatty acid composition and inherent antioxidant compounds. The types of fatty acids present in the oil, and in particular their number of double bonds, determine the type and extent of chemical reactions that may occur during the storage period.

The oxidative deterioration of EVOO can be delayed by employing suitable methods like maintaining cool and stable storage temperature conditions and avoiding light exposure, but oxidation cannot be avoided [19]. Furthermore, EVOO provides a rich source of natural antioxidants. Polyphenols, sterols and fatty acid are present in considerable quantities in olive oil and play a major role in human metabolism, being simultaneously influent in factors such as stability, flavour, and color of the oil [6]. These include carotenoids, tocopherols and phenolic compounds which may act, by different mechanisms, to confer an effective defence system against free radical attack. Some authors have estimated their contribution to oil stability, that of phenolic compounds being around 30%, fatty acids 27%, α-tocopherol 11% and carotenoids 6% [20]. EVOO differ from other edible oils in the abundance of oleic acid (monounsatured), a medium content of palmitic and stearic acids (satured) and a low percentage of polyunsatured fatty acids like linoleic and linolenic. Polyunsatured fatty acids are oxidised faster than monounsatured ones, this explains the higher stability of EVOO in comparison with other vegetable oils where the percentage of polyunsatured fatty acids can reach levels above 40%.

3.2.1 Influence of temperature and oxygen concentration

It is not easy to differentiate the individual effects of temperature and oxygen on the oxidation process as strong interactions exist between them [22]. A temperature higher than 20–22 °C is dangerous because it increases the risk of oil autoxidation. The speed of fatty acids oxidation in the oil depends, in fact, on the storage temperature, which must be controlled between 13–18 °C. The most dangerous risk factor for oxidation of EVOO is however contact with air. During storage, in the presence of oxygen, the oil can be oxidised because of the activity of the lipoxidase enzymes or by a chain reaction due to free radicals. Data derived from Di Giovacchino et al. [23] indicates that the risk of oxidation is greater when the oil occupies only a small part of the container, thus favouring continued solubilisation of oxygen in the oil and, therefore, reactions with fatty acid radicals and the formation of hydroperoxides.

3.2.2 Light exposure

Natural or artificial light exposure is a risk factor in EVOO stability. Light may stimulate some photosensitizers able to produce singlet oxygen which promotes a rapid polyunsaturated fatty acid oxidation.

Chlorophylls and pheophytins have a prooxidant action in the presence of light; they act as catalysts in the formation of singlet state oxygen, which can react directly with the double bonds of oleic, linoleic, or linolenic fatty acids, thereby generating reactive species of oxygen. Thus, chlorophylls and their derived pigments enhance the early phases of the process of autoxidation and generate allyl hydroperoxides. [20]

It has been demonstrated that oils stored in light displayed significantly lower tocopherol, carotenoid and chlorophyll contents than the oils kept in the dark [18]. The oils kept in the dark mainly contained products of primary oxidation, while the oils kept in the light contained products of secondary oxidation, describing light as the main cause for the increase in absorbance at 270 nm and especially for the loss of oil color. In addition, the oils kept in the light showed

significantly higher values of triglyceride oligopolymers which are considered to be good indices of the level of oxidation of edible oils and fats owing to their high stability and low volatility.

As expressed above to reduce the speed of oil oxidation, it is recommended to store the oil in a fully filled container where the volume of air must be below 3–5 % of the total volume, well closed, in the dark and at a temperature between 13 °C and 18 °C.

4. Relationship between color degradation and oxidative deterioration

Lipid oxidation has been acknowledged as the major problem affecting edible oils, as it is the origin of important deteriorative changes in their chemical, sensory, and nutritional properties [24]. As lipids oxidize, they may form hydroperoxides, which are susceptible to further oxidation or decomposition into secondary reaction products such as aldehydes, ketones, acids, and alcohols. In many cases, these compounds adversely affect flavour, aroma, taste, nutritional value, and overall quality [25].

The EVOO used for this autoxidation and color degradation study were selected between 16 spanish olive oils varities cultivated in the west Mediterranean area (Alfafarenca, Arbequina, Arbosana, Carrasqueña, Changlot, Cuquillo, Dotó, Dulce, Farga, Llumero, Menuda, Morruda, Picual, Rojal, Serrana,Villalonga) on the basis of their color degradation due to storage time. In a previous research we found that the varieties mentioned above could be classified according to their color degradation after 3 years of storage in the dark and with a room temperature varing from 18 ºC to 26ºC in 3 groups: group nº 1 encompassed the varieties Arbequina and Cuquillo, group nº 2 Changlot and Menuda, and group nº 3 the rest of oils. Varieties Cuquillo, Farga, Menuda and Serrana have been chosen in this study as a representative sample of each group given their conditions of homogeneity in the group to which they belong

The color differences between fresh oils and oxidized oils are represented in Fig. 1. Values of the chromatic ordinates L*, a* and b* of the selected oils are shown in Table 2. Luminosity values (L*) increased slightly for Cuquillo and Menuda oils while no difference was found in this parameter for Farga and Serrana samples.

The storage appear to have a significant effect on the chromatic ordinates a* and b* for all the tested oils which correspond to the green/yellow zone in CIELAB space. The more negative the value of a* coordinate the greener the color of the oil, and the more possitive the value of b* the yellower the color of the oil. Values of both a* and b* above -10 and below 10 respectively shows oils with very litlle coloration, almost transparent. As we can see in Fig. 1 and Table 2. all oils except Cuquillo presented after 3 years of dark storage values of a* and b* between -10 and 10, that's to say, all samples except Cuquillo have lost the caractheristic color that identifies EVOO. Although there is no general agreement about the visual threshold for a normal observer to appreciate the CIELAB color difference it is ussually considered that changes in oil color could become noticeables between changes of 1 and 3 units. In our study, Farga is the less colored oil with the higher value of a*combined with the lower value of b* (chroma = 5,37), followed by Serrana that reached a chroma value of 7,99, on the other side, Cuquillo presents after the storage a chroma value of 31,33.

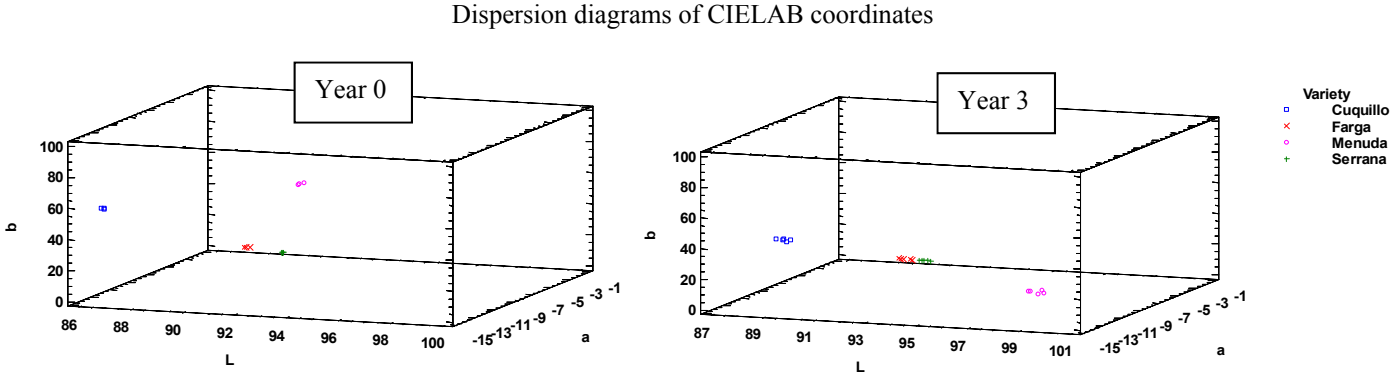


Fig. 1 Dispersion diagrams of CIELAB coordinates (L, a, b) for year 0 (oil extraction year) and year 3 (after 3 years of storage in darkness).

Table 2 CIELAB coordinates for 4 different oil varieties measured in the year of the oil extraction (L_0, a_0, b_0) and after 3 years of storage in darkness (L_1, a_1, b_1) (n = 5; mean value ± standard deviation).

Variety	L_0	a_0	b_0	L_1	a_1	b_1
Cuquillo	86,61 ± 0,09	-14,52 ± 0,07	58,79 ± 0,14	87,40 ± 0,19	-8,72 ± 0,26	30,10 ± 1,48
Farga	89,99 ± 0,12	-8,60 ± 0,03	22,18 ± 0,16	89,88 ± 0,23	-2,54 ± 0,12	4,74 ± 0,42
Menuda	94,20 ± 0,05	-14,51 ± 0,18	81,45 ± 0,11	97,31 ± 0,31	-8,89 ± 0,37	5,50 ± 0,22
Serrana	90,72 ± 0,05	-6,84 ± 0,11	15,51 ± 0,25	90,76 ± 0,17	-5,75 ± 0,02	5,56 ± 0,14

Storage has not only produced a variation of the coloration of the oils but also a change on their chemical composition as the parameters of oxidation demonstrate (Table 3).

The biologically synthesized fat is neutral, that's to say the contained oil in the healthy olive that is in the tree has 0% of free acidity. The presence of free fatty acids is, therefore, a resultant abnormality, among other factors, of the poor state of the fruits, poor treatment, or poor conservation of the same. A very low acid percentage corresponds with a high quality oil. Values close to 0.1 indicate a perfect state of the olive and a correct manipulation of the fruits during the oil extraction. The current regulation considers the limit for EVOO in 0.8º. It is, however, very frequent to find commercial oils below this limit (usually two to five tenths of acidity). As shown on Table 3, all the samples had initially very low values of free acidity expressed as percentage of oleic acid, three years after, all oil varieties except Menuda showed acidity levels below the 0,8º required by the regulation for EVOO. Menuda oil has deteriorate its quality from according to this parameter from EVOO to VOO.

Fats oxidize on contact with oxygen in the air, when a vegetable fat begins to oxidize various compounds are formed; among them are peroxides, which are considered the first oxidation products. The peroxide value determines the primary oxidation state of an oil before the smell and stale taste is appreciated. In our study, after 3 years of storage the peroxide value for the oils of Farga and Serrana varieties are far above the limit of 20 meq of O_2/kg set by de European Union and by the International Olive Oil Council (Table 3), and therefore would not meet official quality standards for EVOO.

K232 value indicates the initial oxidation process of a vegetable oil. As it occurred with the peroxide value, the samples that have shown higher K232 values are Serrana (7,24) and Farga (3,81) with values that exceed the threshold established by the regulation for virgin oils. These data go hand in hand with those obtained for the level of peroxide

K270 parameter detects a more advanced oxidative state. As the oxidative process progresses, the peroxides are modified obtaining components that absorb ultraviolet light at a different wavelength (270 nm) than the hydroperoxides. Following the criterion of K270 value, Serrana was the oil which has suffered more secondary oxidation followed by Farga and Menuda, all of three showed values above the limit indicated by the olive oil regulation after 3 years of storage. Secondary oxidation compounds are usually thought to be the most detrimental to human health [26], which makes K270 one of the most important quality parameters.

Table 3 Quality parameters for 4 different oil varieties measured in the year of the oil extraction (t_0) and after 3 years of storage in darkness (t_1) (n = 5; mean value ± standard deviation).

Variety	Free acidity t_0 (as oleic acid %w/w)	Free acidity t_1 (as oleic acid %w/w)	Peroxide value t_0 (meq O_2/kg oil)	Peroxide value t_1 (meq O_2/kg oil)	K 232 t_0	K 232 t_1	K 270 t_0	K 270 t_1
Cuquillo	0,23 ± 0,01	0,34 ± 0,01	12,63 ± 0,13	20,30 ± 0,21	1,90 ± 0,03	2,37 ± 0,12	0,12 ± 0,01	0,17 ± 0,02
Farga	0,20 ± 0,06	0,56 ± 0,03	4,34 ± 0,06	42,15 ± 1,20	1,57 ± 0,05	3,81 ± 0,09	0,07 ± 0,01	0,24 ± 0,03
Menuda	0,19 ± 0,02	1,19 ± 0,02	4,30 ± 0,08	17,33 ± 0,84	1,59 ± 0,05	2,14 ± 1,31	0,07 ± 0,01	0,23 ± 0,03
Serrana	0,20 ± 0,01	0,61 ± 0,02	4,43 ± 0,21	66,81 ± 0,66	1,50 ± 0,08	7,24 ± 0,09	0,12 ± 0,02	0,35 ± 0,01

Free acidity, Peroxide value, expressed as milliequivalents of active oxygen per kilogram of oil (meq O2/kg), and K232and K270 extinction coefficients calculated from the absorption at 232and 270 nm, respectively, were measured following the analytical methods described in European Regulation EEC 2568/91 and later amendments.

Both polar phenols content and tocopherol have been successfully correlated with olive oil stability [27-28]. In our study, as a result of oxidation, is predictable that oils coming from Serrana and Farga fruits have reduced its polar phenols and tocopherol contents.

By relating the results obtained in the color measurements with the results of the chemical analyzes we can observe a parallelism between the values of a* and b* and the level of primary and secondary oxidation of the EVOO after three years of storage. The most chemically modified EVOO (Farga and Serrana), with higher values of peroxides and K232 and K270 indexes coincide with the oils with lower chroma, higher values of a* and lower values of b*. On the other side, the EVOO of Cuquillo, the only EVOO that still suitable for consumption after three years of storage according to European Regulation, is the EVOO that has presented the higher chroma and higher b* value. No correlation has been found between the increase of the luminosity (L*) and the chemical quality of the EVOO.

The course of time worsens the quality of the EVOO. To delay at maximum the EVOO color degradation and chemical oxidation, it is recommended to store the oil in a fully filled container, well closed, in the dark and at a temperature between 13 °C and 18 °C and consume it before 18 months of bottling.

References

[1] Pérez-Jiménez F. Virgin olive oil: its functional capacity. Molecular Nutrition and Food Research. 2007; 51:1197.

[2] López-Cortés I, Salazar DM, Salazar DC, Velázquez-Mari B. Fatty acid, sterols and polyphenol content in most monovarietal oils of the spanish east areas. In De Leonardis A (Eds.). Virgin olive oil. Product, composition, uses and benefits for man. Nova Science Publishers, Inc, New York. 2014; 41-69.

[3] International Olive Council. Norma comercial aplicable a los aceites de oliva y los aceites de orujo de oliva. COI/T15/NC nº 3/Rev 10.2015; pp 7-9.

[4] Commission Implementing Regulation (EU) No 1348/2013 of 16 December 2013 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis

[5] Ceballos C, Moyano MJ, Vicario IM, Alba J, Heredia FJ. Chromatic evolution of virgin olive oils submitted to an accelerated oxidation test. Journal of the American Oil Chemists´Society. 2003; 80: 257-262.

[6] Roca M, Mínguez-Mosquera MI. Changes in chloroplast pigments of olive varieties during fruit ripening. Journal of Agriculture and Food Chemistry. 2001; 49: 832-839.

[7] Criado M, Moltiva M, Goñi M, Romero M. Comparative study of the effect of the maturation process of the oilve fruit on the chlorophyll and carotenoid fractions of drupes and virgin oils from Arbequina and Farga cultivars. Food Chemistry. 2007; 100: 748-755.

[8] Criado M, Romero M, Moltiva M. Effect of the technological and agronomical factors on pigment transfer during olive oil extraction. Journal of Agriculture and Food Chemistry. 2007; 55: 5681-5688

[9] Gallardo-Guerrero L, Roca M, Mínguez-Mosquera MI. Distribution of chlorophylls and carotenoids in ripening olives and between oil and alperujo when processed using a two- phase extraction system. Journal of the American Oil Chemists´Society. 2002; 79: 105- 109.

[10] Ranalli A, Malfatti A, Lucera L, Contento S, Sotiriou E. Effects of processing techniques on the natural colouring and the other functional constituents in virgin olive oil. Food Reserach International. 2005; 38: 873-878.

[11] Gandul-Rojas B, Gallardo-Guerrero L, Roca M, Aparicio-Ruiz R. Chromatografic methodologies: compounds for olive oil color issues. In: Aparicio R, Harwood J, editors. Handbook of Olive Oil. New York: Springer; 2013. p. 220.

[12] Gandul B, Minguez-Mosquera MI. Chlorophyll and carotenoid composition in virgin olive oils from various Spanish olive varities. Journal of the Science of Food and Agriculture. 1996; 72: 31-39.

[13] Minguez-Mosquera MI, Rejano-Navarro L, Gandul-Rojas B, Sanchéz A, Garrido-Fernández J. Color pigment correlation in virgin olive oil. Journal of the American Oil Chemists´Society. 1991; 68: 332-336.

[14] Moyano M, Meléndez-Martínez A, Alba J, Heredia F. A comprehensive study on the colour of virgin olive oils and its relationship with their chlorophylls and carotenoids indexes (II): CIELUV and CIELAB uniform colour spaces. Food Research International. 2008; 41: 513-521.

[15] Moyano M, Melgosa J, Alba J, Hita E, Heredia F. Reliability of the Bromothymol blue method for color in virgin olive oils. Journal of the American Oil Chemists´Society. 1999; 76: 687-692.

[16] Morelló JR, Moltiva MJ, Tovar MJ, Romero MP. Changes in commercial virgin olive oil (cv Arbequina) during storage, with special emphasis on the phenolic fraction. Food Chemistry. 2004; 85: 357-364.

[17] Cinquanta L, Esti M, Di Matteo M. Oxidative stability of virgin olive oils. Journal of American Oil Chemistry Society. 2001; 78: 1197–1202.

[18] Caponio F, Bilancia MT, Pasqualone A, Sikorska E, Gomes T. Influence of the exposure to light on extra virgin olive oil quality during storage. European Food Research and Technology. 2005; 221: 92-98.

[19] Kristott J. Fats and oils. In Kilcast D, Subramaniam P (Eds.). The stability and shelf-life of food, CRC Press, Boca Raton, Boston, New York & Washington, DC. 2000. p. 279–309

[20] Aparicio R, Roda L, Albi MA, Gutiérrez F. Effect of various compounds on virgin olive oil stability measured by Rancimat. Jounal of Agricultural and Food Chemistry. 1999; 47: 4150–4155.

[21] Giuliani A, Cerretani L, Cichelli A. Chlorophylls in olive and in olive oil: chemistry and occurrences. Critical Reviews in Food Science and Nutrition. 2011; 51:678–690.

[22] Velasco J, Dobarganes C. Oxidative stability of virgin olive oil. European Journal of Lipid Science and Technology. 2002; 104:661-676.

[23] Di Giovacchino L, Mucciarella MR, Costantini N, Ferrante ML, Surricchio G. Use of Nitrogen to Improve Stability of Virgin Olive Oil During Storage. Journal of the American Oil Chemists' Society. 2002;79: 339-344.

[24] Velasco J, Dobarganes C. Oxidative stability of virgin olive oil. European Journal of Lipidic Science and Technology. 2002; 104:661–676.

[25] Matthaeus B. Oxidation of edible oils. In: Foods and beverages and antioxidant applications, vol 2, Woodhead Publishing series in food science, technology and nutrition oxidation.Woodhead Publishing, Oxford/Philadelphia. 2010; pp 183–238

[26] Guichardant M, Lagarde M. Analysis of biomarkers from lipid peroxidation: a comparative study. European Journal of Lipidic Science and Technology. 2009; 11:175–182

[27] Sayago A, Marín MI, Aparicio R, Morales MT. Vitamin E and vegetable oils. Grasas y Aceites. 2007; 58:74–86.

[28] Papadopoulos G, Tsimidou M, Boskou D. Stability of virgin olive oil: Assessment of natural antioxidants and other related factors. In Proceedings of the 7th International Flavor Conference, Developments in Food Science 32. Food Flavors, Ingredients and Composition, June 24-26, 1992, Pythagorion, Samos, Greece; Charalambous G., Ed.; Elsevier: Amsterdam, The Netherlands, 1993; pp 321-326.

Food additives: Colorants

Aybuke Ceyhun Sezgin[1] and **Sibel Ayyıldız**[2]

[1] Gazi University, Faculty of Tourism, Department of Gastronomy and Culinary Arts, Ankara / Turkey
[2] Giresun University, Dereli Vocational School, Giresun / Turkey

Food additives are used to protect food, increase quality and extend shelf life in many stages, from production to consumption of food. Additives used in the food industry are added to the food during preparation, production, packaging and storage stages. Colorants added by food producers to color food or to adjust the color to desired level are among the commonly used food additives. Considering today's developing production technologies, foods fade or discolor at various stages of processing, storage, and sale due to physical and chemical conditions such as heat, light, pH and oxygen. Colorants are used to regain these color losses, to enhance weak colors, to give color to the food that is actually colorless, and to win back the favor of customers by hiding low quality. Colorants are used in the production of soft drinks, candies, bakery products, canned and vegetable products, dairy products, and meat and fish products.

Keywords: food additives; food technology; colorant; E code

1. Introduction

The need of using additives in the food industry arises from technological requirements. In addition, factors such as the increase in the world population, the decrease in raw material resources, and the tendency of people to raise the living standards are affecting technological developments. Different production techniques, diversification of products, increase in the tendency of seasonal foods to be consumed in every period of the year, extension of shelf life of the products and necessity of quality standardization have made the use of food additives compulsory in food sector [1]. The colorant additives used in the food industry are added in order to add color to the food during processing and storage [2-3]. Colorants differ from each other by various physical and chemical properties such as chemical structures, sources and usage purposes. In this paper; information on food additives, colorants and general properties of colorants, usage purposes and health issues is provided.

2. Food additives and their usage purposes

Food additives are added in various stages of food production with two main purposes; one is to make food safe by preventing bacteria growth, oxidation formation and other chemical changes and the other is to improve consumer's taste by enhancing the organoleptic properties such as color, appearance, flavor and smell of the food [4-5] .

Some of the additives are produced from natural sources such as corn, beet and soybean, while others are artificial or synthetic. Nowadays, many consumers prefer to buy convenience food rather than preparing food at home. Convenience food containing additives and preservatives are not spoiled by bacteria and yeast, thus the quality and taste are preserved. There are more than 3000 antioxidants and preservatives having antimicrobial activity in the food industry. Salt and sugar are the most commonly used additives [3]. Spices and sulphites are also additives that have been used since ancient times in order to maintain the desired properties in the food production. With the development of the food industry in the 20th century, the use of new food additives has become inevitable for the production of many convenience foods [6].

Food additives are used in various processed foodstuffs such as non-perishable food, chips, ketchups, sauces, chocolates, puddings, colored candies, powdered drink mix, processed meat and dairy products, canned and fermented products, and instant soups [5]. The International Codex Alimentarius Commission (CAC), established by the joint efforts of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) defines **food additives** as “any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, but added to the food with the intention of achieving a specific purpose, known for having safe dose levels and subjected to legal permission”. Food additives are substances added in the production, processing, preparation, packaging, wrapping, transport, preservation and storage stages [7,4,5-8].

It is known that salt was used for curing meat products in 3000 BC and human used to benefit from salt and wood smoke as food preservation method around 900 BC [7]. In general, additives have functions such as extending shelf life, increasing product quality and variety, making food production quick and easy, reducing costs, and achieving the production standards. Some additives are used to attribute new features to food while some are used to preserve their existing properties [4-5].

If a substance is added to food for a specific purpose, this additive is called the direct additive substance. For example, xanthan gum is a direct additive added to salad dressing, chocolate milk, bakery fillings, puddings. Usually

direct additives are written in the contents of the food label. Indirect food additives are substances that are added to food in trace amount during packaging, storage and other processes [5].

Classification of food additives according to purpose of use [5-9].
1. Extending the shelf life by protecting quality (Preservatives)
Antimicrobial substances: (nitrite, nitrate, benzoic acid, propionic acid, sorbic acid)
Antioxidant substances: (BHA, BHT, gallates)
2. Improving food structure, preparation and cooking
pH regulators
Anti-caking agent (silicate, magnesium oxide, magnesium carbonate)
Emulsifiers (lecithin, mono and diglycerides)
Stabilizers, thickeners, sweeteners
Fermentation agents
Moisture regulators
Maturing agents
Bleaches, fillers, foam conditioners, polishers
3. Improving color and flavor
Flavor enhancers (MSG)
Condiments (flavour substances)
Colorants (tartrazine, indigotine)
Protecting and improving nutritional value (nutritional elements)
Replacing missed nutrients during processing (B_1, B_2, niacin)
Adding nutritional elements that might be lacking in the diet (A, D vitamins)

2.1 Legal regulations on food additives

In order to make the relevant legal regulations it is crucial to classify the additives used in processed food production by analyzing their health conditions and decide whether they are favorable to use or not. For this reason, the legal use of food additives in food is regulated by the joint efforts of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO). The Codex Alimentarius Commission (CAC) was established in 1963 to carry out the joint work of these two institutions. The task of this commission is to regulate the standardization of food-related practices across the world in terms of health and technology. Documents prepared by this commission with this purpose are accepted as a reference for safe food production in all countries of the world. Codex Committee on Food Additivies and Contaminants-CCFAC and Joint Expert Committee on Food Additivies - JECFA are the two institutions working on food additives within this commission [7].

JECFA conducts studies on the safety of additives on human health and prepares lists of additives which are determined to be safe for use at certain doses. These committees investigate all scientific data of the food additives in their agendas, make evaluations, and identify ADI values by various means. The committees examine the food contaminants, naturally occurring toxic substances and veterinary drugs and determine the ADI and maximum residue limits (MRL) [3].

ADI (Acceptable Daily Intake): As a result of long-term and detailed toxicological studies carried out by experts working in the JECFA commission, the acceptable dose of additives for experimental animals is determined. This value for humans is calculated as follows; one mg per kilogram of body weight is determined first and this number is divided by 100, which is accepted as safety factor by the commission, in order to decide on Acceptable Daily Intake. The name of the additive and the maximum acceptable amounts in different food are determined in the lists prepared using this data. ADI is the maximum acceptable consumption amount of an additive per day. ADI values do not differ from country to country as they are stated as international standards. ADI values are not written on food labels. These values are stated in the relevant legislation and should be known by food producers [10].

E code: Each food additive that is permitted for use in the European Union countries is given an "E" code. The "E code" used to identify food additives, inform consumers and prevent any confusion, consists of the letter E of the European Union (EC) and three digit numbers. More than 8.000 food additives are available today. Only 350-400 of them have the "E" code. All chemicals that are defined as additives and used in food, whether natural or synthetic, are within this coding system. Food additives are sometimes written with their original names, sometimes only with "E" codes, sometimes with both. In addition to this code, there are also other internationally recognized numbering systems such as the INS (International Numbering System) or CAS (Chemical Abstract Service) [4, 3-11].

3. Color and colorants in foods

Usually the properties of food such as shapes, colors, tastes, smells and textures are improved to satisfy consumer expectations. Color is one of the most significant factors that directly affects consumers' food choice and eating desires

[12]. Color is a visual feature that arises from the spectral distribution of the light. The formation of light occurs from the interaction of matter and light, and humans see wavelengths between 380-770 nm. As for the other matters in nature, the colors of the food are also based on this basic principle [13]. Color that affects taste recognition and product acceptability might has influence on both actual and perceived nutritional value of food [8].

Color additives are any dye, pigment or substance that are capable of coloring (either alone or by reacting with other substances) when added to food, medicament, cosmetics, or applied to the human body [5]. Today, due to the development of the food industry, the need to colorize food products has increased for various reasons. The Codex Alimentarius Commission (CAC) defines colorants as substances added to color food or to correct the color of the food. In addition, the colorants are added to restore the natural color lost during processing and storage of the food, to enhance the existing color, to strengthen the weak color, to color the food which is actually colorless and to win consumers by hiding low quality [13, 2-3].

Colorants are usually added to processed food such as candies, snacks, margarine, cheese, soft drinks, jam/jelly, gelatin, pudding and pastry fillings [5]. It is known that in medieval ages nitrate was used to enhance the color of meat and to prevent botulism apart from salt and smoke that were used as preservatives. In addition, food colorants were used by Egyptians in 400 BC to regulate the color of wine and confectionery products [7]. Among the natural colorants added to food around the mid-1800s were vegetable-derived products such as saffron, carrot, mulberry and flower, various animal originated pigments, and minerals from copper and iron. The first synthetic dye obtained from organic coal tar was used in butter and cheese around the end of the 19th century [14].

3.1 Classification of colorants

Directives on colorants are examined in 3 groups. These are as follows:

1. Colorants whose ADI values are determined and allowed for use,
2. Colorants permitted to use only for special purposes (such as surface finishing) ($CaCO_3$, aluminum, silver, gold),
3. Colors that are only allowed to use in some foods (Titanium dioxide, vegetable carbon, red beet)

The use of colorants outside of this classification is banned. Furthermore, despite the ban on the addition of colorants to products such as mineral waters, milk, flour and tomato paste, different implementations are applied in national legislation [15]. Colorants differ from each other by various properties such as chemical structures, sources, and purpose of use. As it is difficult to classify the colorants according to these properties, they are divided into two groups based on their sources as natural and synthetic [13].

3.1.1 Natural colorants

The use of natural colorants is known to date back 2600 BC and written records have been found in China. It has been reported that food colorants were used in Europe during the Bronze age [16]. Natural food colorants, which continue to be used worldwide and known to have significant benefits when consumed, are demanded by people for their long or short-term effects as well as for their reliability, functionality, biological potential and health effects. Consumers perceive natural colorants as safer than to synthetic colorants which are thought to be harmful [12].

Many consumers associate good and natural looking food and drinks with high quality while they think the other way around when it comes to faded and artificial shining products. In addition, the production of colorants from known sources such as beetroot, grape, cabbage and paprika makes the consumer feel safe and makes it easier to familiarize and accept the product [17]. Natural colorants are less stable to heat, light or pH, and their production is inadequate to meet industrial demand. They quickly fade when exposed to light and shows low resistance to acidity and high temperature. For example, Annatto turns to pink from yellow at low pH and chlorophyll turns to brown from green [15]. This makes natural origin colorants more expensive. For example, natural red and yellow colorants may cost 100 times more than synthetic products with the same effect [8]. Natural coloring matters are synthesized by plant and animal organisms or microorganisms and they naturally exist in them. Pigments produced by modification of living organism materials such as caramel, vegetable carbon and Cu-chlorophylline (vide infra) are accepted as natural although they are not found in nature (except carbon). Nature identical colors are man-made pigments found in nature. Carotene, canthaxanthin and riboflavine are nature identical colors [2]. The most notable colorants obtained from animal sources are Natural Sepia (cuttle fish), Crimson (Kermes Louse) and Tyrian purple (Murex shellfish) [16].

3.1.1.1 Organic natural colors

Anatto: Annatto is one of the oldest known natural carotenoids used as food colorant. Tropical annatto is a pigment derived from the pericarps of *Bixa orellana L.* tree seeds [18]. Having yellow-orange food colorant property, annatto is used in smoked fish, various beverages, bakery products, and dairy industry. [19]. It has also been reported that annatto is used especially in cheese, butter, margarine and snacks [20].

Anthocyanins: Anthocyanins are natural polyphenolic pigments group responsible for various colors of many fruits and vegetables ranging from red to blue. Typically, the most common sources of anthocyanins used in food industry are grape, elderberry and blackcurrant. Anthocyanins are also found in red beets, black carrots and so forth [17].

Anthocyanins are not only vegetative as plant roots, flowers, leaves and plant matrices are. Algae/microalgae, fungi/yeast and aquatic animals are also used as raw material to extract carotenoid pigments [12].

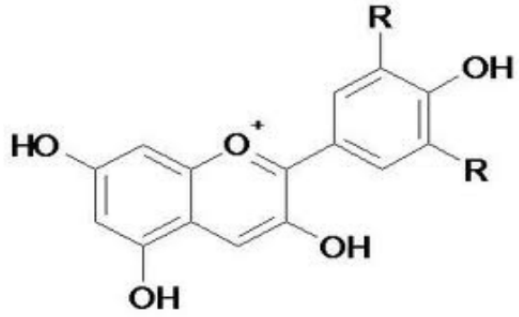


Fig. 1 Chemical structure of Anthocyanins [16].

B-Apo-8'Carotenal and Carotenoic Acid Ethyl Ester: Existing in the form of crystals or powder crystals and having a color of dark and red violet with a metallic lustre [13], they are used in the production of soft drinks, confectionary, coatings, soups, sweets and sauces [4].

Vegatable Carbon (Vegetable Black): Carbo vegetabilis or carbon black is the only natural color that provides shades of black or gray. It is used in candies, ice-creams and frozen sorbet [17].

Caramel: Caramels constitute more than 80% of all food colorants and are classified according to their production methods [19]. The distinctive taste, odor and the amber color that come out when heated is called caramel. Although not obtained from plants or animals, the caramel produced by heating the sugars is used in various food products. Sufficient pressure and temperature conditions must be provided during the caramel production process [21].

Carotenes: As they are added to foods with high fatty acid content, carotenoids are preferred in various food production. The main coloring substance of carotenes, β-carotene, is yellow and red [12].

Chlorophyll: Chlorophyll is common substance in nature which is a green pigment occurring as a result of vegetable and fruit plants photosynthesis [22]. Chlorophyll is used in bakery products, dairy products, candies, cereals, jams and jellies to give green color. Chlorophyll is also used as a complementary color when it is needed to dim off the yellowish cheese milk color [17].

Carmine (Natural Red 4): Dactylopius coccus (Cochineal) is a local insect in South America and Mexico. The pigment obtained from this insect and its egg is carminic acid [16]. Carmine is a compound that carminic acid creates with aluminum pigment. Since it is an expensive substance as a color additive it is not economical to use in food industry. Carmine is used to give pink color in dragee coatings and protein food as the use of FD&C pigments is unfavorable for protein food [23].

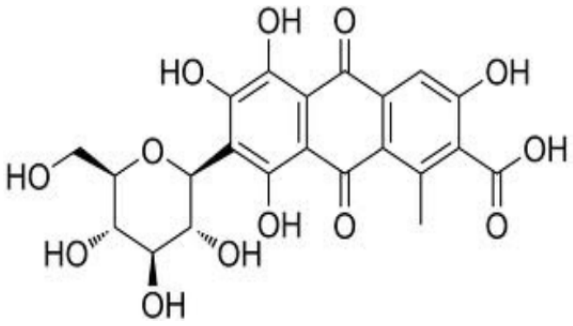


Fig. 2 Chemical structure of Carmine [16].

Sepia officinalis L. (Female Cuttlefish): This pigment, called Sepiaxnthine, has a concentrated orange-red color. It is used as colorant and sweetener in pasta and sauce production. Usually, the ink of the cuttlefish is used in black pasta production. The most important colorant sources obtained from animals are Natural Sepia, Crimson (from Kermes Louse) and Tyrian purple [16].

Curcumin (Turmeric): Turmeric is a plant that is cultivated in many tropical countries, especially Curcuma longa and India. Curcumin is the main coloring pigment of turmeric which is used as a spice for thousands of years and is one of the main components in curry sauce [17]. Curcumin is mainly used in dairy products, beverages, cereals, mustard, food concentrates, pickles, sausages, confectionery, ice cream and bakery products. Mixed with annatto, it is also added to the seasonal sauces, mayonnaise sauces and butter [16-21].

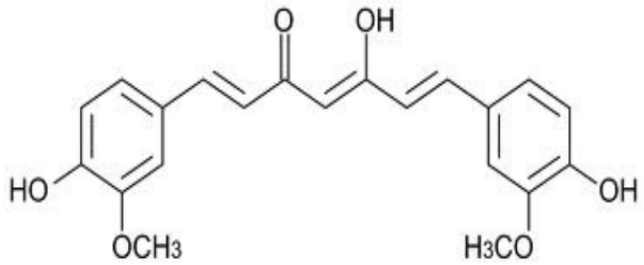


Fig. 3 Chemical structure of Curcumin [24].

Lycopene and Lutein: Lycopene, the main coloring substance of red tomato, is a carotenoid class colorant. This substance is dark red and viscose. Lutein, another colorant in the carotenoid class, is the main coloring substance of xanthophylls and gives yellow color [13].

Beetroot Red: The color pigments exist in the redbeet are red and yellow pigments. Redbeet is used in the production of ice cream, dairy products, jams and jellies and give strawberry color in confectionery that are not exposed to high temperature [16].

Paprika Extract: The important pigments found in red pepper are a mixture of capsanthin and capsorubin. Both are carotenoids responsible for the red color of the dye [19].

Riboflavin: Riboflavin (vitamin B_2), used as a yellow food colorant, is banned in most countries. It is used in many food products such as sorbet, various drinks, sweets and ice cream. Riboflavin is preferred in the production of cereal-based products, but its use is limited due to its mild odor and naturally bitter taste [2].

Monascus purpureus: Red pigments produced by the fungus named *Monascus purpureus* are used in traditional products in some countries due to their various properties. The use of these pigments has not yet been legally regulated in the European Union, the United States, Brazil, the Philippines and Taiwan. This pigment, which is soluble in water, has been used in confectionery and red rice wine production in Japan for many years. Additionally, this pigment has cholesterol-lowering properties [16].

3.1.1.2 Inorganic natural colorants

Aliminium dust and silver for silver gray color, *gold* for real gold color, *iron oxides* for yellow, red, brown or black colors, *titanium dioxide* for white color and *calcium carbonate* for opaque appearance are important inorganic natural colorants [13]. These colorants are used in the production of confectionery coating, liqueur decoration, chocolate, calcium carbonate, gum and bread [4].

3.1.2 Synthetic colorants

The substances which are not found in nature due to chemical structures and obtained by chemical synthesis are known as synthetic colorants [1]. The first synthetic organic color obtained is the purplish lilac color discovered by William Henry Perkin in 1856. It was obtained from the organic coal tar. Synthetic colorants have many advantages over natural colorants. Synthetic food colorants surpass natural colorants due to their high coloring ability, various color tone, homogeneous color distribution, brightness, stability, and ease of application [14]. With high water and oil dissolution properties, shelf life of the synthetic colorants are quite long. Chemical solubility matters when making a classification. Synthetic colorants are divided into three groups according to their solubility; [13].

1- Water soluble synthetic colors
2- Fat soluble synthetic colors
3- Lake colors

3.1.2.1 Water soluble synthetic colors

Allura Red AC: This synthetic colorant, generally known to be derived from insects, is actually produced from coal tar. Allura Red AC is used in the production of food like carbonated drinks, gums, snacks, sauces, soups, wine and especially apple wine. While European Union affirms its use; Denmark, Belgium, France, Switzerland, Austria, Norway and Sweden have banned it [3].

Amaranth: This substance gives reddish brown color and it is water soluble [13].

Sunset Yellow: Sunset yellow, which is orange red color, is usually used for food such as bread, drinks, cereals, sweet powders, ice cream and snacks [8].

Brilliant Blue FCF and Brilliant Black BN: Available in blue and black colors, it exists in powder and granular form. It is easily soluble in water while being less soluble in ethanol [13]. Brillant black is used in the production of various cheese, wine, sauce and beverages [12].

Tartrazine: Tartrazine is used to obtain lemon yellow color and is added to food products such as bread, beverages, cereals, peanuts, confectionery, cream, ice cream and canned food [8-5].

Erythosine: Being a xanthen-class colorant in the structure of benzoate, erythosine exists the form of red powder or granules [13]. It is added to flavored milk and puddings, ice products, chewing gum and candies, jelly and drink powders [15].

Quinoline yellow: Quinoline yellow is a synthetic substance used to obtain a greenish yellow color. It is used in soft drinks, jams and canned foods, edible ice, sweets, candies, pickles, sauces and spices [4].

Brown FK and Brown HT: Brown FK is used in smoked and cured fish, meat and chips, while Brown HT is used in various biscuits, chocolates and cakes [12].

Other water soluble synthetic colorants are Green S, Indigotine, Patent Blue V, Litolurubin BK, Red 2G, Ponso 4R and Azorubin [13].

3.1.2.2 Oil soluble synthetic colorants

Artificial colorants soluble in oil or organic solvents are insoluble in water as they do not contain groups capable of forming salt form as in water-soluble colorants. This group of colorants are not allowed to be used for food coloring because of their toxic properties. For example, the use of oil-soluble Penso SX for the coloring of butter and margarine was banned in 1976. Oil Red XO, Yellow AB used in the coloring of orange peels and Yellow OB are not allowed to use because of their toxic properties [13].

3.1.2.3 Lake colorants

Lake colorants are water-insoluble precipitation of aluminum hydrate substrate and are produced in the form of very fine powders. The dye content and particle size determine the color tone of the powder [25]. As they are not soluble in water, oil and other solvents, they are dispersed in food and produce color. They are used in cakes, biscuit fillings, confectionery, powder drinks, sweets, soups and spice mixtures [13].

3.2 Colorants used in food industry

The European Union has identified 43 colors and each of which is given an E number as a food additive. Of these, 17 are synthetic pigments and 26 are natural pigments [25]. Natural colors have always been part of the nutrition. Chlorophylls, carotenoids and anthocyanins are consumed with the food we eat every day [14]. Coloring of food with natural resources is thought to be healthier than coloring with synthetic dyes. For this reason, natural color formulations need to be developed for the use of food additives such as emulsifiers, carriers and antioxidants [26].

Non-alcoholic beverages: The non-alcoholic beverage industry has a significant share in the sales of food colorants. The coloring of soft drinks makes drinks more attractive and increases consumer perception of the overall fruit content and quality. All coloring categories such as synthetic, nature identical, natural and caramel colors (for coke) are used in non-alcoholic beverages [25]. In addition, Quinoline Yellow, Sunset Yellow FCF, Orange Yellow S and Brilliant Blue, banned in many European countries, are also used in non-alcoholic beverages [3]. Penso 4R, Brown HT, Brilliant Blue FCF, Green S, Quinoline Wax, Indigo Karmin are also used in soft drinks. While synthetic colorants are used in many fruit flavored soft drinks, cola and beer are colored with caramel [13]. Sunset Yellow is used in orange juice, Tartarizine in lemon juice, Penso 4R in cherry juice, Carmoisein and Sunset Yellow in strawberry juice, Carmoisin and Penso 4R in raspberry juice [15]. Allura red and Brillant Black are used in wine production [12-3].

Sugar products: Usually Amarant, Penso 4R, Allure Red AC, Sunset Yellow FCF, Tartarizine and Karmoicine as well as Brown HT, Black PN, Brilliant Blue, Patent Blue, Erythrocin and Indigo Karmin are used in confectionery products. The amount of colorant added depends on the preferred color tone. Excessive coloring causes non-attractive dull color. The use of Lake colorants is recommended to use in chewing gums, bonbons and dragées in order to make synthetic colors leave color on mouth [13]. In addition, inorganic natural colorants such as Gold, Silver, Aluminum powder and Iron oxides are used in confectionery surface coating, chocolate and liquor decoration [4].

Bakery products: Colorants are widely used in dough products, biscuits, cake creams and coatings. Caramel and carbon black are used in combination with synthetic colorants. Caramel is crucial in the coloring of rye bread. Allure Red AC, Sunset Yellow FCF, Brown HT, Tartarizine, Penso 4R are the most preferred colorants in baked products [13]. Tartarizine, Penso 4R, Sunset Yellow FCF are also used in chocolate cake, breakfast snacks, plain cakes and wafers [15].

Canned food and vegetables: The colorants to be added to canned food must be resistant to sterilization or high cooking temperature and acidic environment conditions. Amarant, Penso 4R, Allure Red AC, Sunset Yellow FCF, Red 2G and Indigo Karmin are the most commonly used synthetic colorants in canned fruit. Anthocyanins, β-carotene, Carminic acid and Chlorophyll are natural colorants used in canned fruits and vegetables [13].

Dairy products: The addition of liquid dyes to ice cream is done after pasteurisation. Some cheeses and butter are colored with β-carotene and anatto [15]. In addition Sunset Yellow FCF, Penso 4R, Indigo Carmine, Erythrocin, Tartarine, Amarant and Allure Red are also frequently used in dairy products. Almost all kinds of ice cream are added with synthetic colorants. Sunset Yellow FCF and Tartarissin color mixtures are widely used in ice cream cones [13]. Sunset Yellow FCF and Carmosine are used in yogurt production [3].

Meat and fish products: Carmosine, Erythrocin, Tartaricin, Allure Red AC and Red 2G are widely used colorants in meat and fish products. Water-soluble colorants suitable for pickling conditions are required for fish products produced by curing or smoking. For these practices, it is stated that Brown FK is particularly favorable, and in some cases mixtures of Carmosin, Tartarizin and Sunset Yellow FCF are also used [13].

3.3 Legal regulations on colorants

Today, in all countries of the world, food additives and especially colorant-related regulations are in focus. Moreover, despite global cooperation and harmonization efforts, these regulations vary from country to country [27]. The European Food Safety Authority (EFSA) and the Food and Drug Administration (FDA) are the most important regulatory bodies authorized to protect and improve human health, as well as to ensure the quality and safety of food products [12].

The FDA has primary legal liability for determining and regulating the safe use of food additives. Food manufacturers must first get approval from the FDA to use a new colorant in food production [5]. In developed countries, the use of colorants in the food industry depends on a number of toxicity tests (such as detection of the acute, subchronicand chronic toxicity, carcinogenicity, mutagenicity, teratogenicity, reproductive toxicity, accumulation in the body, bioenergy effects and immune effects) [28]. Currently, 16 natural color pigments and synthetic origin 9 color pigments including lutein are allowed to use in European countries but vegetable carbon, aluminum, silver and gold, chlorophyllsand and chlorophyllins and calcium carbonate are not allowed in the USA [27]. The natural colorants

permitted to use are betaines-betaines, quinones-cocineal, flavonoids-anthocyanins, isoprenoids-carotene, annatto (bixin, norbixin), red pepper extract, lutein, canthaxanthin, porphyrins-chlorophylls chlorophyllin of these compounds and copper complexes, caramels and curcumin [21].

To distinguish food colorants from other colorants, an FD&C number is issued by the FDA to the colorants permitted under the Food, Drug and Cosmetic Act. Similarly, the European Union has also given the E code number to the colorants allowed to use. For this reason, in some cases there might be 3 different code numbers for the same colorant in the literature. For example, the CI number is 15985, the FD&C number is Yellow 6, and the E code is E110 for Sunset Yellow [13].

3.4 Colorants in health aspects

Different views emerge when the food additives are assessed in terms of health risk. Most people think that food safety regulations are inadequate for consumer protection [29]. Consumers see naturalness as an important property and natural foods are considered to be safer and even healthier than artificial food [30].

It is known that children are always interested in foods and drinks with vibrant colors. For this reason, adding attractive colors to the food is thought to increase the taste and appetizing properties of food and drinks for consumers [31]. However, it has long been argued that synthetic food colorants and other food additives have an adverse effect on children's behavior. The effects of these substances can manifest as behavioral disorders, hyperactivity and attention deficits that show significant individual differences on children [32, 33-28]. In a study conducted, it is reported that Brilliant Blue, Tartrazine FD&C Yellow No 5 cause hyperactivity disorder [3]. In addition, six of the most common synthetic food colorants that are reported to have negative effects on attention deficit and hyperactivity are tartrazine (E102), quinolone yellow (E104), sunset yellow FCF (E110), carmoisine/azorubine (E122), Ponceau 4R (E129) and Allura Red AC (E129). These products are especially exist in sugary products and beverages [12]. Especially Allura Red AC can cause cancer, chromosomal aberration, developmental toxicity, DNA damage, genotoxicity, hyperactive behavior in children, neurotoxicity, psychotoxicity, reproductive toxicity [34].

In recent years, an increase in the incidence of allergies and asthma has been observed in humans, and this increase has been associated with food additives, especially with colorants [28]. Synthetic food additives have been shown to increase the urticaria and asthma in some individuals [35]. It has been reported Allura Red, one of the colorants, increases asthma and urticaria while Tartarin causes asthma and migraine [3].

In addition to the negative effects of synthetic colorants on health, colorants that affect health in a positive way are also available. The positive relationship between health and carotenoids has been found by the discovery of β-carotene, an important pigment in skin protection and cell growth [19]. Lycopene, a natural pigment found in tomatoes, is effective in reducing all types of cancer, especially the risk of breast, prostate and cervical cancer [25-24].

It has been determined that curcumin is a powerful antioxidant and protects against oxidative damage in cellular components and is effective in the treatment of wounds and burns. Curcumin has been found to prevent cancer formation and progression, increase the activity of certain enzymes responsible for digestion, promote detoxification of liver which acts as an antibacterial agent and even have anti-HIV properties [25-36]. Antioxidant activity of carotenoids, having coloring property, pre substance of vitamin A, protects against oxidative damage and is evaluated positively for health [25].

4. Conclusion

Today, factors such as increase in food production variety, diversification of technological developments and changes in consumer nutrition habits have increased the number of processed foods. In many countries, the use of food additives has been compulsory to ensure the quality characteristics of processed foods and to extend shelf life. In particular, the coloring additives used in color formation, which plays an important role in consumer preferences, are added to give color to the food and to win the consumers. Colorants are used in many food industries, such as the production of soft drinks, confectionery, bakery products, canned and vegetable products, dairy products and meat and fish products. When evaluated in terms of health aspects; the use of additives must be performed within the limits and to fulfill a certain function within the legal framework.

References

[1] Altuğ T, Gıda katkı maddeler. In: Altuğ T, editor. Gıda katkı maddeleri. Meta Basım, İzmir; 2006. p. 1-16.
[2] Aberoumand A. A review article on edible pigments properties and sources as natural biocolorants in foodstuff and food industry. World Journal of Dairy & Food Sciences. 2011; 6 (1): 1-78.
[3] Pandey RM, Upadhyay SK. Food additive. InTech. İndia. 2012; 5:1-31.
[4] Emerton V, Choi E. Essential guide to food additives. 4th ed. Cambridge UK: Leatherhead Publishing. 2008.

[5] FDA. Food and Drug Administration, & US Department of Health and Human Services. Food ingredients and colors. International Food Information Council (IFIC) and US Food and Drug Administration. 2010 [cited 2017 June 19]. Available from: https://www.fda.gov/downloads/Food/FoodIngredientsPackaging/UCM094249.pdf.
[6] Chaudhary NK. Food additives. Bibechana. 2010; 6:22-26.
[7] Luckey TD. Introduction to food additives. In: Furia TE, editor. Handbook of food additives. USA: CRC Press LLC. 2nd edition; 1968. p. 1-27.
[8] Branen AL, Haggaerty RJ. Introduction to food additives. In: Branen AL, Davidson PM, Salminen S, Thorngate J, editors. Food additives. CRC Press; 2001. p. 1-11.
[9] Akbulut M, Gıda katkı maddeleri: fonksiyonları ve kaynakları. 1. Ulusal Helal ve Sağlıklı Gıda Kongresi; 2011 November 19-20; Ankara.
[10] Food and Agricultural Organization (FAO). Guidelines for the Simple Evaluation of Dietary Exposure to Food Additives CaC/GL 3-1989. Adopted 1989. Revision 2014 (formerly Guidelines for the Simple Evaluation of Food Additive Intake). Codex Allimentairus International Food Standarts. 2014 [cited 2017 June 19]. Available from: www.fao.org/input/download/standards/6/cxg_003e.pdf
[11] Varela P, Fiszman SM. Exploring consumers' knowledge and perceptions of hydrocolloids used as food additives and ingredients. Food Hydrocolloids. 2013; 30:477-484.
[12] Martins N, Roriz CL, Morales P, Barros L, Ferreiral ICFR. Food colorants: challenges, opportunities and current desires of agro-industries to ensure consumer expectations and regulatory practices. Trend in Food Science. 2016:1-72.
[13] Demirağ K, Uysal V. Renklendiriciler In: Altuğ T, editor. Gıda katkı maddeleri. Meta Basım, İzmir; 2006. p. 169-191.
[14] Griffiths JC. Coloring foods & beverages. Foodtechnology. 2005; 59(5):38-44.
[15] Karaali A. and Özçelik B. Gıda katkı maddeleri, gıda katkısı olarak kullanılan doğal sentetik boyalar. Gıda Dergisi. 1993; 18(6):389-396.
[16] Lakshmi GC. Food coloring: the natural way. Research Journal of Chemical Sciences. 2014; 4(2):87-96.
[17] Nielsen SR, Holst S. Developments in natural colourings. In: MacDougall DB, editor. Colour in food improving quality. Woodhead Publishing Limited and CRC Press LLC. Cambridge England. 2002. p. 331-352.
[18] Satyanarayana A, Rao PP, Rao DB. Influence of source and quality on the color characteristics of annatto dyes and formulations. Food Science and Technology. 2010; 43:1456-1460.
[19] Solymosi K, Latruffe N, Morant-Manceau A, Schoefs B. Food colour additives of natural origin. In: Scotter MJ, editor. Colour additives for foods and beverages. Woodhead Publishing, Oxford, 2015; 3-34.
[20] Giuliano G, Rosati C, Bramley PM. To dye or not to dye: Biochemistry of annatto unveiled. TRENDS in Biotechnology. 2003; 21(12):513-516.
[21] Janiszevska-Turak E, Pısarska A, Krolczyk JB. Natural food pigments application in food products. Nauka Przyroda Technologie. 2016; 10-4 #51:1-13.
[22] Özcan M, Akgül A. Gıdalar için doğal renk maddeleri 1. Gıda. 1995; 20(4):365-369.
[23] Saldamlı İ. Gıda kimyası. Hacettepe Üniversitesi Yayınları, Ankara.1998; 112:489-495.
[24] Chengaiah B, Rao KM, Kumar KM, Alagusundaram M, Chetty CM. Medicanal importance of natural dyes. International Journal of PharmTech Research. 2010; 2(1):144-154.
[25] Downham A, Collins P. Colouring our foods in the last and next millennium. International Journal of Food Science and Technology. 2000; 35:5-22.
[26] Scotter M. Emerging and persistent issues with artificial food colours: Natural colour additives as alternatives to synthetic colours in food. Quality Assurance and Safety of Crops & Foods. 2011; 3:28-39.
[27] Lehto S, Buchweitzb M, Klimmb A, Straßburgerb R, Bechtolda C, Ulbertha F. Comparison of food colour regulations in the EU and the US. Food Additives & Contaminants. 2017; 34(3):335-355.
[28] Amchova P, Kotolova H, Ruda-Kucerova J. Health safety issues of synthetic food colorants. Regulatory Toxicology and Pharmacology. 2015; 73(3):914-922.
[29] Tarnavölgyl G. Analysis of consumers attitudes towards food additives using focus group survey. Agriculturae Conspectus Scientificus. 2003; 68(3):193-196.
[30] Bearth A, Cousin ME, Siegrist M. The consumer's perception of artificial food additives: influences on acceptance, risk and benefit perceptions. Food Quality and Preference. 2014:1-39.
[31] Saleem N, Umar ZN, Khan Sİ. Survey on the use of synthetic food colors in food samples procured from different educational institutes of Karachi city. The Journal of Tropical Life Science. 2013; 3(1):1-7.
[32] McCann D, Barrett A, Cooper A, Crumpler D, Dalen L, Grimshaw K, Kitchin E, Lok K, Porteous L, Prince E, Sonuga-Barke E, Warner JO, Stevenso J. Food additives and hyperactive behaviour in 3-year-old and 8/9-year-old children in the community: a randomised, double-blinded, placebo-controlled trial. The Lancet. 2007; 370:1560-1567.
[33] Larsen JC. Legal and illegal colours. Trends in Food Science & Technology. 2008; 19:S64-S69.
[34] Sabnis RW, Pfizer I, Madison NJ. Biolojical dyes and stains, synthesis and industrial applications. Wiley Publication. Canada. 2010:1-521.
[35] Pollock I, Warner JO. Effect of artificial food colours on childhood behaviour archives of disease in childhood. 1990:65-74.
[36] Beaglehole R, Bonita R, Maqnusson R. Global cancer prevention: an important pathway to global health and development. Sydney Law School Legal Studies Research Paper. 2011; 11(77):1-29.

Food analysis: From structure, chemistry and flavour to *foodomics*

A. Vilela[1], **T. Pinto**[2], **B. Gonçalves**[2], **E. Bacelar**[2], **A.C. Correia**[3], **A.M. Jordão**[3] and **F. Cosme**[1,*]

[1] CQ-VR, Chemistry Research Centre, University of Trás-os-Montes and Alto Douro, School of Life Sciences and Environment, Department of Biology and Environment, Enology Building, 5000-801 Vila Real, Portugal. email avimoura@utad.pt

[2] CITAB, Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro, School of Life Sciences and Environment, Department of Biology and Environment, 5000-801 Vila Real, Portugal. email tpinto@utad.pt; bertag@utad.pt; areale@utad.pt

[3] Polytechnic Institute of Viseu (CI&DETS), Agrarian Higher School, Estrada de Nelas, Quinta da Alagoa, Ranhados, 3500-606 Viseu, Portugal. email antoniojordao@esav.ipv.pt; anacorreia@esav.ipv.pt

* Corresponding author: email fcosme@utad.pt

Food analysis, in the 21st century, demands the development of more robust, efficient, sensitive, and cost-effective analytical methods to assure safety, quality, and traceability of food according to legislation and consumers' demands. Computer vision is a fast, non-invasive low-cost method for evaluating food quality. Regarding to food composition, analysis by instrumental methods such as chromatographic, spectrophotometric, gravimetric, fluorimetric, rheological, mass spectrometric and more recently NIR and FTIR technologies are available. Food flavour analysis, using a variety of techniques, has been conducted for many years to help in the development of new products, to study shelf-life, and to maintain food quality. Flavour analysis usually takes one of two forms, sensory or instrumental. The measurement for the sensory aspects of flavour or aroma is descriptive sensory analysis, using a trained sensory panel and a number of sensory lexicons have been published recently. Thus, this chapter presents a review of analytical techniques used for food analysis, namely for structure, composition, sensory evaluation and a brief overview on *foodomics*.

Keywords: Chemometric approaches; chromatographic analysis; consumer behavior; microscopy; sensory analysis

1. Food analysis, from flavour to *foodomics*

The major factors that influence consumer's perception of food quality are the external appearance and the flavour compounds which are food aroma and taste. Understanding the nature of flavour compounds and their effects on human sensory responses is important and flavour analysis is an intimate part of the process.

While taste can basically be characterised as being salty, sour, sweet [1] bitter/astringent [2], *umami* [3] or fat – fat taste [4], the sensations caused by aroma compounds are considerably more complex. The number of volatile and semi-volatile compounds in foods ranges from 50 to 250 compounds in fresh fruits and vegetables to more than double of these numbers in thermally processed foods such as roast beef [5, 6]. Moreover, humans may or may not have the ability to differentiate these odorous constituents. The odour-active compounds are usually found in very low concentrations - hundred parts per million (ppm) for strongly flavoured foods to less than 10 ppm for weakly flavoured foods. In addition, many of these flavour compounds are highly reactive and thermally labile [6]. Thus, food analysis are continuously requesting the development of more robust, efficient, sensitive, and cost-effective analytical methodologies to guarantee the safety, quality, and traceability of foods in compliance with legislation and consumers' demands. From the so-called "wet chemistry" used at the beginning of the 20th century [7], current powerful instrumental techniques are now being used in food laboratories. This improvement has led to significant enhancements in analytical accuracy, precision, detection limits, and sample throughput, thereby expanding the practical range of food applications [8]. The modern global food distribution system heavily relies on food analysis as a tool for new product development, quality control, regulatory enforcement, and problem-solving. Several methodologies have been developed to cope with the complexity and to resolve practical problems related to food analysis.

1.1 Sample preparation and quantification techniques

Schematically, sample preparation can be performed in two basic steps: (i) extraction of target analytes; (ii) removal of interfering substances. Only occasionally can food samples be analysed directly: in most cases they need a sample clean-up step, necessary to remove interfering substances. Sometimes, this becomes a necessary step to make the analysis itself possible, as in the case of samples that need to be treated with derivatizing agents (e.g. methylation of free fatty acids prior to Gas Chromatograph - GC analysis).

Thus, sample preparation is one of the key steps for the development of any analytical method and an effective sample preparation is essential for achieving good analytical results, especially if analytes of interest are present in complex matrices. Advances in sample preparation aim to minimize laboratory solvent use and hazardous waste production, save employee labour and time, and reduce the cost per sample, while improving the efficiency of the analytes isolation [8] without compromising the integrity of the extraction process. Sample preparation methods include liquid-liquid extraction (LLE), solid-phase extraction (SPE) and solid-phase microextraction (SPME). SPME is a very

simple and efficient, solvent less sample preparation method, started in 1989 by Pawliszyn [9] and is ideally suited for coupling with mass spectrometry (MS). According to Pollnitz et al. [10], this technique resulted in clear chromatograms, but with lower sensitivity than obtained from organic solvent extracts. However, in other cases, the proposed SPME strategies were not suitable for the analyses of the most polar compounds, in particular from wine [11]. For example, using the SPME extraction followed by the volatile compounds analysis by gas chromatography coupled to mass spectrometry (GC-MS), Fernandes et al. [12] analysed grape leave extracts infusions prepared from vine leaves collected 30 and 60 days after grape harvest of two Portuguese red grape varieties (Touriga Nacional and Tinta Roriz). Using this analytical technique these authors showed that the volatile compounds existing in vine leaves infusions were dependent on the harvest time, with significantly higher volatile concentration in vine leaves collected after 30 days of harvesting. They identified twelve volatile compounds in vine leaves infusions (6-methyl-5-hepten-2-one; 1-undecene Z-citral; cedrenol; DL-limonene; 2,4-hexadien-2-ol; benzaldehyde; heptane-1,2,4,6-tetraene; cyclohexene 1-methyl-4-(1-methylethyl); 1,6-octadien-3-ol; 3,7-dimethyl; 5,9-undecadien-2-one-6,10-dimethyl-(5E) and α-cedrol) (Figure 1A). Previously, Jordão et al. [13] studied the volatile composition by SPME of oak wood used in cooperage for barrel making and it was possible to quantify a great number of volatile compounds as a result of the different oak wood species analysed.

Anthocyanins are the phenolic compounds responsible for the colour of red wines. For the analysis of the red wine anthocyanin profile using high performance liquid chromatography with diode array detector (HPLC-DAD) no sample preparation is necessary. Anthocyanins from wine obtained from *Vitis vinifera* grape varieties are all monoglycosides, and glucose is attached to the 3-hydroxyl group of the aglycone [14]. In addition, the glucosides of the anthocyanins can be acylated at position 6 of the sugar, with organic acids (acetic, *p*-coumaric or caffeic acid) which are linked to anthocyanin glycosyl units through an ester bond. Anthocyanins in wines obtained from *Vitis vinifera* grape varieties occur as 3-*O*-glucosides, from an anthocyanidin (malvidin, delphinidin, peonidin, petunidin or cyanidin), being the anthocyanin, malvidin-3-*O*-glucoside the most abundant [15] (Figure 1B). Consequently, malvidin is also the reddest individual anthocyanidin [16]. The hydroxylation pattern of the B-ring of anthocyanins is important for their colour and stability. Some anthocyanins are more stable than others, depending on their molecular structure. The increased hydroxylation of the aglycone stabilized the anthocyanidin, therefore trihydroxylated anthocyanins (delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, and malvidin-3-*O*-glucoside) are more stable than dihydroxylated ones (cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside) [17]. Also, cyanidin, delphinidin and petunidin which enclose the *O*-diphenol structure on the B ring are more sensitive to oxidation, since the adjacent hydroxyl groups of *O*-diphenols are more sensitive to enzymatic and non-enzymatic oxidation to create *O*-diquinones, or even *O*-diphenol dimmers [16]. Methoxylated as well as acylated anthocyanins are also more stable [17].

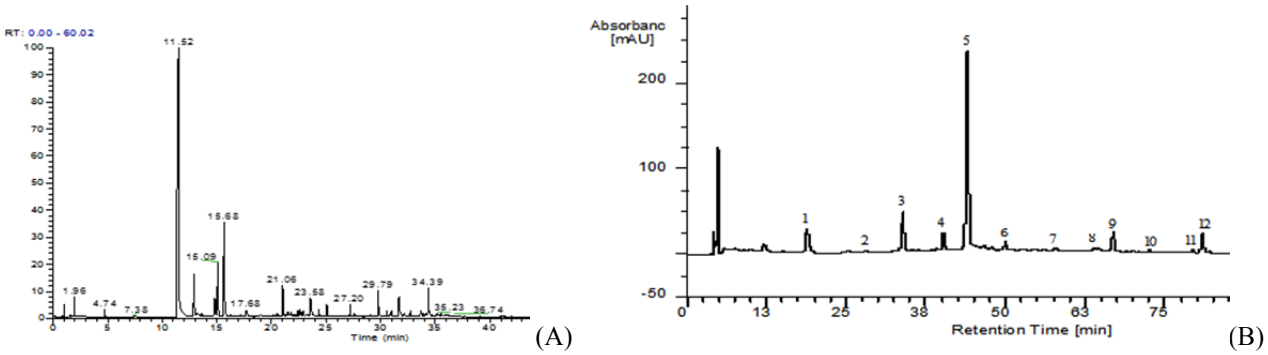


Fig. 1 Chromatogram representing the volatile profile obtained from vine leaves infusions using SPME-GC/MS (A) and chromatogram representing the anthocyanin profile obtained from red grape extracts using HPLC-DAD by direct injection (B).

1.2 Recent food analysis techniques

Computer vision (CV) is a multi-disciplinary field in which many of the supporting technological areas are developing rapidly, such as computer science, artificial intelligence and mechanical engineering and its application in industry has increased considerably in recent years. In fact, CV is a fast, non-invasive low-cost method for evaluating food quality [18]. Image processing systems can be used to several food and agriculture products such as meat, bakery products, dairy products, vegetables, cereals but especially in fruits. Mainly investigated topics are size and shape based classification, colour, texture, defects detection, microbial safety, quality grading and variety determination [19, 20].

Some of the automatic inspection techniques of food products are based on the analysis of images acquired in the visible spectrum but also on images obtained by ultraviolet induced fluorescence (UVIF) spectrum or acquired in the near infrared (NIR) spectrum. There are four standard measurement modes for the acquisition of NIR spectra from a sample: transmission, reflection, transflection, and interaction (interactance). The selection of measurement mode depends on many factors like sample type that could be classified into three groups by the state of the sample: (i) liquid samples, (ii) ground and relatively small solid samples, and (iii) relatively large samples that require nondestructive or non-invasive measurement [21].

Recently, artificial neural networks (ANNs) have been introduced in food analysis [22]. ANNs are a set of mathematical methods, which attempt to imitate the functioning of the human brain [23]. They consist of sophisticated non-linear computational tools that are capable of modelling extremely complex functions. There are some examples of usage of ANNs for olive oil classification according to geographical origin, year of production, merceological category, adulteration, processing, and blending. Generally, in those works ANNs have been built using only one kind of analytical data, such as data obtained through mass spectrometry [24], NIR [25], electronic sensors [26], NMR [27, 28], or traditional standardized methods [29, 30].

Due to the increasing ease in which data can be obtained, data fusion is an expanding trend. The main goal is to optimize the information obtained in order to exploit the synergies in individual information provided by the different techniques (Figure 2) [31].

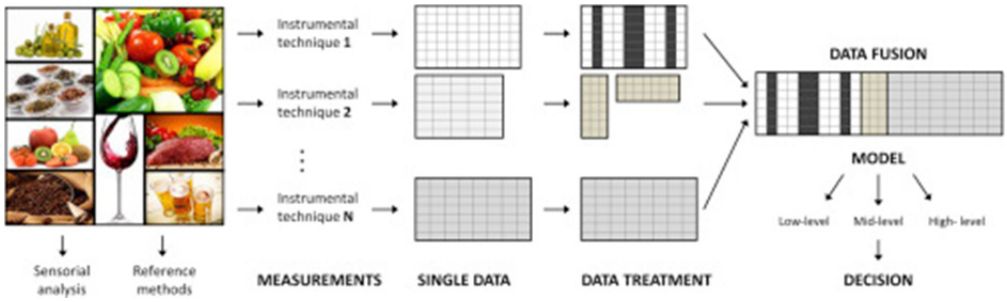


Fig. 2 Data fusion scheme. Adapted from Borrás et al. [31].

Other advanced techniques are the hyperspectral-image analyses which are also very important since they allow researchers to expand the scope of the inspection of food products to their internal quality characteristics. Many approaches and applications have shown the usefulness of hyperspectral imaging in the food industry. For example, in recent years, hyperspectral imaging is used as a method to evaluate the state of the fish freshness, for apple bruise detection, for quality inspection of citrus fruits and to predict the sugar content distribution in melons [32].

2. Microscopy techniques

The history of food microscopy dates back to 1850, when Arthur Hassall used a microscope to distinguish coffee from chicory [33]. Hassal's results were crucial and used by other authors in the ensuing years. Nowadays, the great concern with quality control due to new market restrictions has become so important that it has demanded a technology of process geared toward more reliable tests and new methods of monitoring product quality [18]. Microscopy techniques vary in method of image production, resolution and type of signal detected, and give a particular type of structural information that is unique to the technique used.

Studies suggested that foods having similar structures can be loosely grouped together as foods that have similar textures. Most foods have biological origin, but are processed to several degrees, sometimes to such an extent that their biological origin is not readily apparent, for example, grain *vs*. bread, muscle *vs*. salami or milk *vs*. cheese. Visual changes due to processing (e.g. milling of grain, gelatinization of starch, and comminution of meat, heat denaturation of proteins, gelation of milk and proteolysis of proteins) are the results of changes at the microscopic and molecular levels. Imaging techniques can be used to help evaluate such changes in terms of morphology and composition [34]. On the other hand, there are an increased interest in the role that some nutrients play in preventing or ameliorating the effect of major diseases (for example, some types of cancer, cardiovascular diseases, eye disorders, among others). In this respect, the bioavailability or the proportion of an ingested nutrient that is made available (that is, delivered to the bloodstream) for its intended mode of action is more relevant than the total amount present in the original food. Disruption of the natural matrix or the microstructure created during processing may influence the release, transformation, and subsequent absorption of some nutrients in the digestive tract [35], Figure 3.

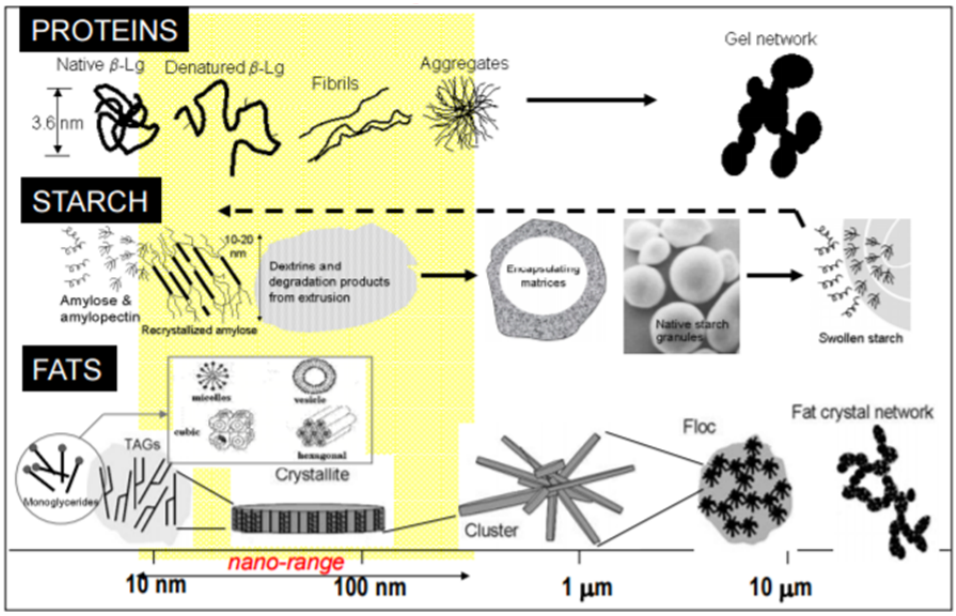


Fig. 3 Some structuring mechanisms of food components. Adapted from Aguilera [36].

Different imaging techniques are now available for microscopic determination of food components. Among the most widely used are light microscopy (LM) to study the microstructure, transmission electron microscopy (TEM), as well as scanning electron microscopy (SEM) with higher magnifying (imaging) powers. To study the ultrastructure of foodstuff it can also be used the confocal laser scanning microscopy (CLSM) [37]. Other techniques, such as atomic force microscopy (AFM), ultrasound and magnetic resonance imaging (MRI) are used for specific food applications. The most commonly used method is LM with transmitted light in the visible spectrum of light. Thus, the microscopes used in LM are composed of a beam of visible light (photons) that represents the illumination source, a system for focusing the source onto the sample, a location to place the sample and the objectives and oculars [34].

Although the magnification of this equipment is reduced if compared with the electronic microscopes, the LM works with samples prepared by specific or basic staining to differentiate the individual internal structures [37]. The basic staining is normally used to enhance all structures present in the final product, which are then identified by their morphological characteristics (shape, size and the mutual cell configuration, the presence of crystals, grains, or other elements). The CLSM is a rather new technique for structural analysis of biological and food material. In contrast to conventional light microscopy, the light source is replaced by laser, a scanning unit and a pinhole in the back focal plane, which improves the limited depth of focus. The primary value of the CLSM to research is its ability to produce optical sections through a three-dimensional (3-D) specimen, for example a piece of tissue or other thick objects [38].

Since in electron microscopy the illumination source is a focused beam of electrons, the resulting micrographs cannot be in colour but various shades of gray. Colour can be distinguished thanks to the differences in their affinity for various heavy metals such as osmium and uranium. Electron microscopy can be divided into SEM and TEM. These two types of electron microscopes differ from each other in the way in which the image is formed. The TEM works exactly as a light microscope, allow electrons to transmit through the material forming a two-dimensional image, whereas the SEM presents 3D-looking images of the specimen surface, since the electron beam only sweeps the surface of the sample, not through it. Thus, in TEM, the samples must be very thin, with 50-100 nm, while samples for SEM can be thicker, because the SEM visualizes the surface of three-dimensional objects.

LM, SEM and TEM can be used to put in evidence different aspects of particulate structures. For example, in a study on microporous, particulate gels [39], LM was used to visualize pores, TEM was applied to evaluate particle size, and SEM allowed to detect how the particles were linked together, that is, the three dimensional structure. CLSM represents a suitable alternative method in food microstructure evaluation because it requires a minimum sample preparation. Indeed, the examination of the three-dimensional structure of the protein network of pasta samples [40], or high fat foods [41] that cannot be prepared for conventional microscopy techniques, without the loss of fat, can be prepared, with success, for CLSM technique [42], Figure 4.

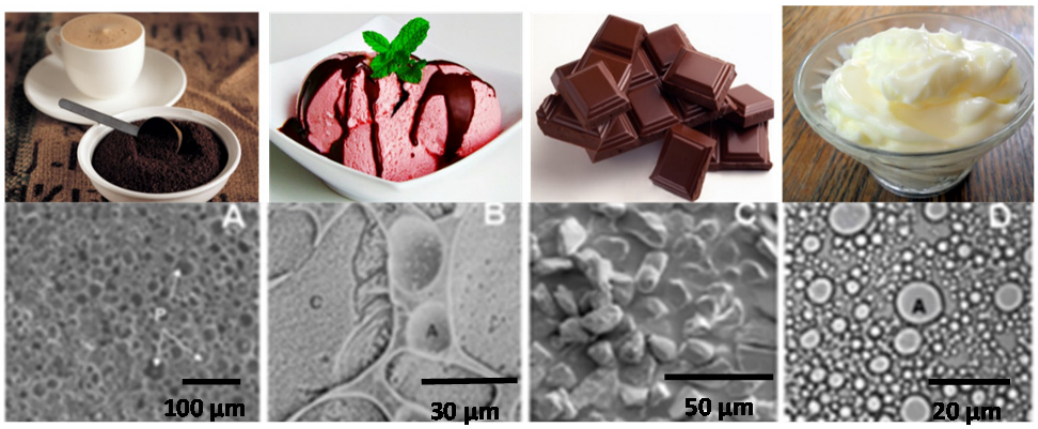


Fig. 4 Visualizing food structures in scanning electron microscopy (SEM). A- Microporous structure of soluble coffee; B- Micro-structures in ice cream; C- Micro-structures in chocolate; D- micro-bubbles of air in mayonnaise. Adapted from Aguilera [36].

3. Foodomics

The application of "omics", the new science, called *foodomics* is an interesting new approach for performing food analysis. *Foodomics* studies the food domain as a whole with the nutrition domain to reach the main objective, the optimization of human health and well-being. In the past 20 years, *omics* technologies presented a great development in different fields such as genomics, transcriptomics, proteomics, and metabolomics. An emergent variety of *omics* sub-disciplines (epigenomics, lipidomics, interactomics, metallomics and diseasomics) has also appeared. With the *omics* approach, researchers are now facing the possibility of connecting food components, foods, diet, individual, health, and diseases. However, this broad vision needs the application of advanced technologies, and the ability of looking at the problem with a different approach, a "*foodomics* approach". *Foodomics* has been previously defined as a discipline that studies the Food and Nutrition domains through the application of *omics* technologies [43]. In this context, nutrigenomics and nutrigenetics have been considered as a part of the more general *foodomics* term [44]. *Foodomics* is the comprehensive, high-throughput approach for the exploitation of food science in the light of an improvement of human nutrition.

Since 2009 that f*oodomics* received the interest of scientists, with different cultural backgrounds. This phenomenon occurred at the time of the first international conference about *foodomics*, held in Cesena, Italy (foodomics.eu). The purpose of the conference was to promote a multidisciplinary environment where specialists in *omics* sciences were invited to contribute to the holistic definition of food and to trace a possible way to exploit this view in the nutrition field.

The knowledge that metabolic pathways may be altered in individuals with genetic variants in the presence of certain dietary exposures offers great potential for personalized nutrition advice [45]. MicroRNAs profiling and genome-wide association studies have also contributed. Since nutritional effects of complex diets emerge only if dietary assessments are validated, nutrimetabolomics offers the validation tools on the basis of food intake biomarkers [45].

Nowadays, *foodomics* constitute one of the most relevant and fast developing areas in food science. The worlds of food technology, microbiology, nutrition, genomics, proteomics, glycomics, phosphoproteomics and other methods dealing with posttranslational modification of proteins; and metabolomics, have started to interact. The aim of this cooperation is to improve the food quality and safety, and to prevent food adulteration [46]. The rights of consumers and genuine food processors in terms of food adulteration and fraudulent or deceptive practices in food processing are set out in the European Union regulations regarding food safety and traceability. This system was developed in order to: (1) encourage diverse agricultural production; (2) protect product names from misuse and imitation; (3) help consumers by giving them information concerning the specific character of the product [47]. The potential use of *foodomics* in their development pathway for food production, assessing the safety, originality and quality is shown in Figure 5.

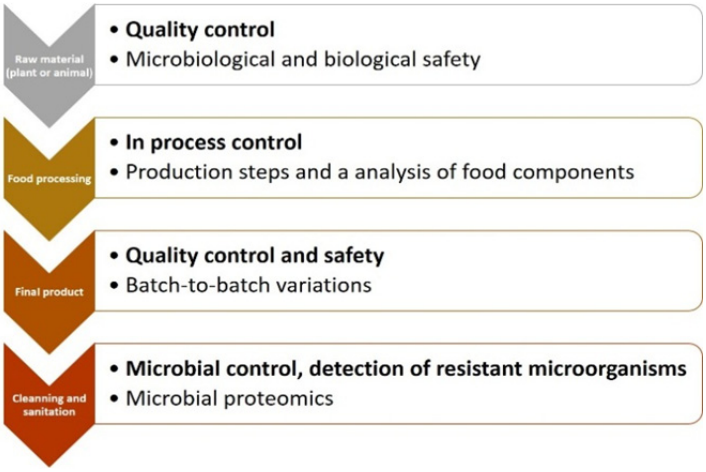


Fig. 5 Use of *foodomics* in the development pathway for food production and assessing food safety, originality and quality. Adapted from Gašo-Sokač et al. [48].

4. Sensory and instrumental flavour analysis

As it was mentioned before, the primary measurement for the sensory aspects of flavour or aroma in food is descriptive sensory analysis, typically with trained sensory panels. Descriptive sensory analyses are used for quality control, for the comparison of product prototypes to understand consumer responses in relation to products' sensory attributes, and for sensory mapping and product matching [49]. This technique may also be used to track product changes over time with respect to understanding shelf-life and packaging effects, to investigate the effects of ingredients or processing variables on the final sensory quality of a product, and to investigate consumer perceptions of products.

In descriptive sensory analyses are always used a sensory lexicon. A huge number of sensory lexicons have been published recently, for diverse food products, from drinks - such as Pink Port Wines [50], coffee [51], and pomegranate juice [52], to different king of food, from cereals like rice [53] to processed tomatoes [54].

A flavour lexicon is a simple set of words used to describe the flavour of a product. A lexicon is then applied during descriptive sensory analysis techniques and provides a source list to describe a category of food products. Although the descriptive panel generates its own list to describe the product array under study, a lexicon provides a source of possible terms with references and definitions for clarification [50].

A well-developed flavour lexicon is also suitable for scientific inquiry. There are three things about the lexicon that are fundamentally different from other sensory evaluation tools [51]: (1) It is descriptive and it doesn't have categories for "good" and "bad" attributes, nor does it allow for ranking food quality. It is purely a descriptive tool, which allows us to say with a high degree of confidence that a food tastes or smells like X, Y, or Z; (2) it is quantifiable. It should allow us not only to say that, for example, a given food has vanillin flavour or aroma, but that it has vanillin at an intensity of 4 on a 5-point scale [50, 55]. This methodology allows us to compare differences among foods with a significantly higher degree of precision; (3) it is replicable. When is used properly by trained sensory professionals the same food evaluated by two different people—no matter where they are, what their prior taste experiences is, what culture they originate from, or any other difference among them—will achieve the same intensity score for each attribute.

According to Lawless and Heymann [1], descriptive sensory tests are amongst the most sophisticated tools in the arsenal of the sensory scientist that use flavour lexicons. The qualitative aspects of a food product include aroma, appearance, flavour, texture, aftertaste and sound properties, which distinguish it from others. Sensory judges then quantify these product aspects in order to facilitate description of the perceived product attributes [1].

A major strong point of descriptive analysis is its ability to allow relationships between descriptive sensory, instrumental and consumer preference measurements. Knowledge of "desired composition" allows for product optimisation and validated models between descriptive sensory and the relevant instrumental and/or preference measures are highly desirable and increasingly, are being utilised within the food industry [56].

Nowadays, there are several different methods of descriptive analysis, including the Flavour Profile Method [57, 58], Texture Profile Method [59, 60], Quantitative Descriptive Analysis™ [61], the Spectrum™ method analysis [62, 63], Quantitative Flavour Profiling [64], Free-choice Profiling [65, 66] and generic descriptive analysis [56]. Reviews of descriptive analysis have been published by several authors, since 1965 to 2014 [56, 63, 67].

A number of different techniques, ranging from conventional solvent extraction and distillation to the newly developed direct thermal desorption (DTD) and solid-phase microextraction (SPME) are available for isolating flavours from diverse food systems. However, instrumental flavour analysis will not give a flavour isolate truly representative of the flavour profile in the food. Sensory techniques, coupled with a technique known as gas

chromatography/olfactometry (GCO), (Figure 6), make it possible to elucidate the relative impacts of various volatiles on the flavour characteristic of a food [68]. CharmAnalysis and aroma extract dilution analysis (AEDA) are also two popular methods to measure the potency of flavour components [68]. However, these two methods are inconsistent with psychophysical views. An alternative method named *Osme* overcomes this problem. It is a quantitative bioassay method used to measure the response to odorants on a time–intensity scale. For accurate quantification, a stable isotope dilution assay (IDA) should be used to target specific compounds [6].

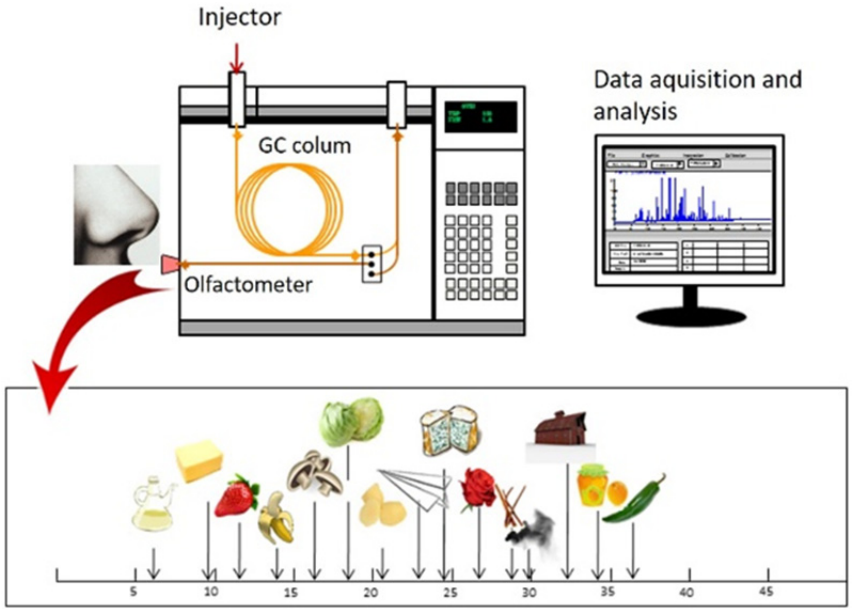


Fig. 6 Gas Chromatography-Olfactometry (GCO) is an analytical tool that uses the human nose as a chromatographic detector in parallel with a conventional one, providing simultaneously both instrumental and sensory results. Therefore, the odour-active compounds among the whole volatile fraction of a sample can be detected. Adapted from iSens [69].

4.1 *e*-noses

It was in the 80s that artificial olfaction sensor technology had its beginnings, with the invention of the first gas multisensor array in 1982 [70]. Advances in aroma-sensor technology, biochemistry, electronics, and Artificial Intelligence (AI) made it possible to develop devices capable of quantify and characterize volatile aromas/flavours released from a multitude of foods and food products. These devices, known as *e*-noses (electronic noses), were planned to mimic human olfactory system, within an instrument able to obtain repeatable measurements, while eliminating operator fatigue [71]. Hundreds of different prototypes of *e*-noses devices have been developed to discriminate complex vapour mixtures containing many different types of Volatile Organic Compounds (VOCs) [72, 73]. So, in accordance with biological odour sensing system, an *e*-nose, which mimics the perceptional mechanisms of biological olfaction, is defined as the instrument that involves an array of various types of electronic chemical gas sensors and a pattern recognition system to detect and distinguish odours in complex samples [47, 74].

An *e*-nose system consists of three major parts: Sample Delivery System - a multisensory array; a detection system - an information-processing unit such as an Artificial Neural Network (ANN); and a computing system - software with digital pattern-recognition algorithms, and reference-library databases [75], Figure 7.

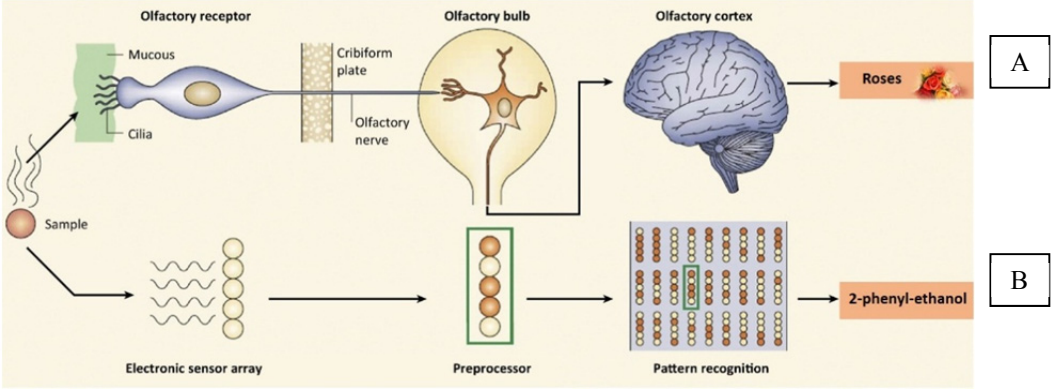


Fig. 7 Analogy between the biological (A) and *e*-nose systems (B). Adapted from Turner and Magan [76].

The *e*-nose sensors look like the primary neurons with different sensitivity to different odours (Figure 7). Through chemical interactions between odour compounds and the sensors, a change in either physical or chemical properties takes place, giving rise to electrical signals which are recorded by a computer system. Consequently, the signals from the individual sensors, involved in the system, show a pattern unique to the compound used and is, afterwards, analysed using chemometric tools. When the sensor patterns for a series of samples are compared, the acquired differences can be correlated with the perceived sample odour or aroma [77-79].

4.2 *e*-tongues

Electronic tongues (*e*-tongues) have been used to obtain data for sourness, bitterness and astringency for foodstuffs such as beers and wines [80, 81]. This accomplishment involved detecting polyphenols and predicting sensory attributes of bitter, sweet, sour tastes and fruity, caramel, artificial, burnt flavours, intensity and body (mouth-feel) using potentiometric/amperometric chemical sensors along with the same pattern recognition techniques described for *e*-nose technology [82]. Taste sensation/perception is the result of physico-chemical interactions of food molecules with a complex system of hundreds of cell buds located randomly all over the tongue [1, 2]. The principle for the *e*-tongue is to combine signals from specific, non-specific and overlapping sensors with pattern recognition. There are four classes of amperometric sensors: metal, conducting polymer, phtalocyanine film and biosensors [83], Figure 8.

Parra et al. [84] developed, for wine, a custom-designed *e*-tongue with a hybrid sensor array based on phtalocyanines, perylene derivatives and conducting polymers; voltammetric electrodes modified chemically with different electro-active substances (polymerized aqueous solution of pyrrole using six doping agents). With this sensor they were able to discriminate and recognize among 12 Spanish red wines based on denomination, origin, grape variety and vintage.

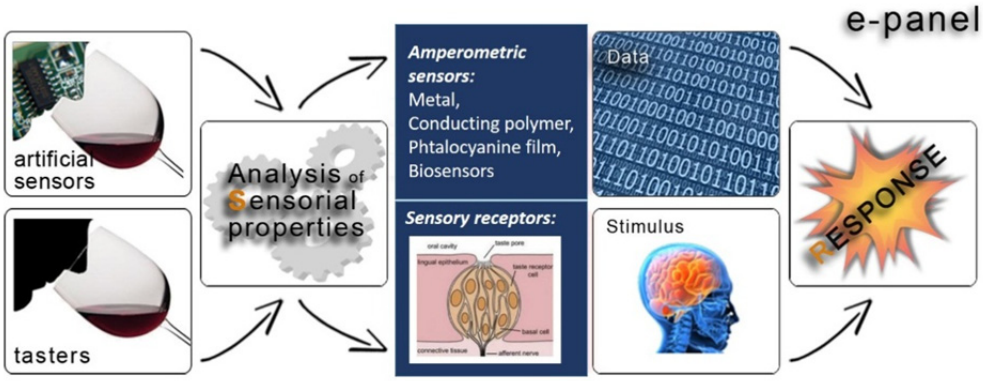


Fig. 8 Schematic representation of a comparison between the transduction mechanism of human taste receptor cells and amperometric sensors of an *e*-tongue. *E*-tongue allows analysing taste responsible compounds such as sugars, acids, alcohols, polyphenols and glutamate. Adapted from iSens [69].

5. Final remarks

Food is a complex and a heterogeneous mixture of vastly numerous and diverse biochemical substances. Thus, food products are analysed for a variety of reasons: compliance with legal and labelling requirements, detection of adulterations, determination of nutritive value, composition and safety, product quality including sensory profile, consumer acceptability and also research and development for new products manufacture. In this context, food analysis is constantly in progress and its concepts have driven change during the last decades from classic analytical methods (titrimetric or gravimetric analysis) to new innovative methodologies by the use of instrumental and biochemical methodologies (such as chromatography, biosensors and spectroscopy) because of the new qualitative and quantitative information provided. In addition, the new consumers' tendencies also induced an improvement of the sensory methodologies. Another challenge is the development of rapid and non-destructive analytical techniques in industrial process and laboratories to promote advances in food analysis.

Thus, despite the availability of modern techniques of separation and identification and also to describe the sensory properties showed in this chapter, rarely is it possible to load a syringe with a food sample, and directly analysis it into an analytical instruments to obtain a sensible result, because of the diversity of food products and the variety of analytical methods depending on the intended application, procedures for preparation of different food sample categories need to be developed, evaluated as an integral part of any analytical methodology.

So, this whole area of food analysis methods will be constantly evolving, trying to meet the increasing demands of producers, the certification and regulation institutions, and also of course the requirements of the market, and in particular the increasingly consumers demanding and informed decision making .

References

[1] Lawless HT, Heymann H. Sensory evaluation of foods: Principles and practices, 2nd ed.; Chapman & Hall: New York, USA, 2010; pp. 1-18, 125-148. 2.

[2] Vilela A, Jordão AM, Cosme F. Wine phenolics: looking for a smooth mouthfeel. SDRP Journal of Food Science and Technology. 2016; 1(1): 1-8.

[3] Vilela A, Inês A, Cosme F. Is wine savory? Umami taste in wine. SDRP Journal of Food Science and Technology. 2016; 1(3): 1-6.

[4] Besnard P, Passilly-Degrace P, Khan NA. Taste of fat: A sixth taste modality? Physiology review. 2016; 96: 151-176.

[5] Fisk ID, Kettle A, Hofmeister S, Virdie A, Kenny JS. Discrimination of roast and ground coffee aroma. Flavour. 2012; 1: 14. http://www.flavourjournal.com/content/1/1/14.

[6] Yong C, Chi-Tang H. Flavor analysis in food. Encyclopedia of Analytical Chemistry. John Wiley & Sons, Ltd. 2006.

[7] McGorrin RJ. One hundred years of progress in food analysis. Journal of Agricultural and Food Chemistry. 2009; 57(18): 8076-8088.

[8] Cifuentes A. Review article: Food analysis: Present, future, and foodomics. International Scholarly Research Network. ISRN Analytical Chemistry. 2012; Article ID 801607, 16 pp. doi:10.5402/2012/801607.

[9] Vás G, Vékey K. Solid-phase microextraction: a powerful sample preparation tool prior to mass spectrometric analysis. Journal of Mass Spectrometry. 2004; 39: 233-254.

[10] Pollnitz AP, Jones GP, Sefton MA. Determination of oak lactones in barrel-aged wines and in oak extracts by stable isotope dilution. Journal of Chromatography A. 1999; 857: 239-246.

[11] Francioli S, Guerra M, López-Tamames E, Guadayol JM, Caixach J. Aroma of sparkling wines by headspace-solid phase microextraction and gas chromatography/mass spectrometry. American Journal of Enology and Viticulture. 1999; 50, 404-408.

[12] Fernandes B, Correia AC, Cosme F, Nunes FM, Jordão AM.. Volatile components of vine leaves from two Portuguese grape varieties (*Vitis vinifera* L.), Touriga Nacional and Tinta Roriz, analysed by solid phase microextraction. Natural Product Resarch. 2015; 29(1): 37-45.

[13] Jordão AM, Ricardo-da-Silva JM, Laureano O, Adams A, Demyttenaere J, Verhé R, De Kimpe N. Volatile composition analysis by solid-phase microextraction applied to oak wood used in cooperage (*Q. pyrenaica* and *Q. petraea*) - Effect of botanical species and toasting process. Journal of Wood Science. 2006; 52, 514-521.

[14] Brouillard R. Chemical structure of anthocyanins. In: Anthocyanins as Food Colors. Markakis, P. (ed.), Academic Pres, Inc., New York, 1-40, 1982.

[15] Ribéreau-Gayon P. Anthocyanins of grape and wines. In: Anthocyanins as Food Colors. Markakis P. (ed.), Academic Press Inc., New York, 209-242, 1982.

[16] Jackson RS. Wine science principle and applications, 3rd ed. Academic Press: Burlington, VT, USA, 2008

[17] Dao LT, Takeoka GR, Edwards RH, Berrios JDJ. Improved method for the stabilization of anthocyanidins, Journal of Agricultural and Food Chemistry. 1998; 46: 3564-356.

[18] Rafiq A, Makroo HA, Sachdeva P, Sharma S. Application of computer vision system in food processing- A Review. International Journal of Engineering Research and Applications. 2013; 3(6): 1197-1205.

[19] Lorente D, Aleixos N, Gómez-Sanchis J, Cubero S, García-Navarrete OL, Blasco J. Recent advances and applications of hyperspectral imaging for fruit and vegetable quality assessment. Food Bioprocess Technology. 2012; 5(4): 1121-1142.

[20] Turgut SS, Karacabey E, Küçüköner E. Potential of image analysis based systems in food quality assessments and classifications. Foodbalt. 2014; 8-12.

[21] Alander JT, Bochko V, Martinkauppi B, Saranwong S, Mantere T. A review of optical nondestructive visual and near-infrared methods for food quality and safety. Journal of Spectroscopy. 2013; 1-36.

[22] Funes E, Allouche Y, Beltrán G, Jiménez A, Review A. Artificial neural networks as tool for control food industry process. Journal of Sensor Technology. 2015; 5: 28-43.

[23] Zupan J, Gasteiger J. Neural networks: A new method for solving chemical problems or just a passing phase? Analytica Chimica Acta. 1991; 248: 1-30.

[24] Goodacre R, Kell DB. Pyrolysis mass spectrometry and its applications in biotechnology. Current Opinion in Biotechnology. 1996; 7: 20-28.

[25] Marini F, Magrì AL, Bucci R, Magrì AD Use of different artificial neural networks to resolve binary blends of monocultivar Italian olive oils. Analytica Chimica Acta. 2007; 599: 232-240.

[26] Cosio MS, Ballabio D, Benedetti S, Gigliotti C. Geographical origin and authentication of extra virgin olive oils by an electronic nose in combination with artificial neural networks. Analytica Chimica Acta. 2006; 567: 202-210.

[27] García-González DL, Mannina L, D'Imperio M, Segre AL, Aparicio R. Using 1 H and 13C NMR techniques and artificial neural networks to detect the adulteration of olive oil with hazelnut oil. European Food Research and Technology. 2004; 219: 545-548.

[28] Rezzi S, Axelson DE, Héberger K, Reniero F, Mariani C, Guillou C. Classification of olive oils using high throughput flow 1 H NMR fingerprinting with principal component analysis, linear discriminant analysis and probabilistic neural networks. Analytica Chimica Acta. 2005; 552: 13-24.

[29] Marini F. Artificial neural networks in foodstuff analyses: Trends and perspectives, a review. Analytica Chimica Acta. 2009; 635: 121-131.

[30] Silva SF, Anjos CAR, Cavalcanti RN, Celeghini RMDS. Evaluation of extra virgin olive oil stability by artificial neural network. Food Chemistry. 2015; 179: 35-43.

[31] Borrás E, Ferré J, Boqué R, Mestres M, Aceña L, Busto O. Data fusion meyhodologies for food and beverage authentication and quality assessment. A review Analytica Chimica Acta. 2015; 891: 1-14.

[32] Hu W, Sun Da-W, Pu H, Pan T. Recent developments in methods and techniques for rapid monitoring of sugar metabolism in fruits. Comprehensive Reviews in Food Science and Food Safety. 2016; 15(6): 1067-1079.

[33] Pospiech M, Lukášková XR, Tremlová B, Randulová Z, Bartl P. Microscopic methods in food analysis. Maso International Brno. 2011; 1: 27-34.
[34] Kaláb M, Allan-Wojtas P, Miller SS. Microscopy and other imaging techniques in food structure analysis. Trends in Food Science and Technology. 1995, 6 (6): 177-186.
[35] Parada J, Aguilera JM. Food microstructure affects the bioavailability of several nutrients. Journal of the Science. 2007; 72 (2): R21-32.
[36] Aguilera JM. Travel to the interior of foods. Presented at Advances in Molecular Structuring of Food Material. Universidade de São Paulo (USP), Pirassununga, Brasil. 2013.
[37] Autio K, Salmenkallio-Marttila M. Light microscopic investigations of cereal grains, Doughs and Breads. LWT- Food Science and Technology. 2001; 34(1): 18-22.
[38] Dürrenberger MB, Handschin S, Conde-Petit B, Escher F. Visualization of food by confocal laser scanning microscopy (CLSM). LWT- Food Science and Technology. 2001; 34: 11-17.
[39] Langton M, Aström A, Hermansson A. Texture as a reflection of microstrure. Food Quality and Preference. 1996; 7(3/4): 185-191.
[40] Fardet A, Baldwin PM, Bertrand D, Bouchet B, Gallant DJ, Barry JL. Textural image analysis of pasta protein networks to determine influence of technological processes. Cereal Chemistry. 1998; 75(5): 699-704.
[41] Wendin K, Langton M, Caous L, Hall G. Dynamic analyses of sensory and microstructural properties of cream cheese. Food Chemistry. 2000; 71(3): 363-378.
[42] Autio K, Laurikainen T. Relationship between flour/dough microstructure and dough handking and baking properties. Trends in Food Science and Technology. 1997; 8: 181-185.
[43] Capozzi F, Bordoni A. Foodomics: a new comprehensive approach to food and nutrition. Genes and Nutrition. 2013; 8(1): 1-4. http://doi.org/10.1007/s12263-012-0310-x.
[44] Cifuentes A. Food analysis and foodomics. Journal of Chromatography A. 2009; 1216: 7109. doi: 10.1016/j.chroma.2009.09.018.
[45] Bordoni A, Capozzi F. Foodomics for healthy nutrition. Current Opinion in Clinical Nutrition and Metabolic Care. 2014; 17(5): 418-424. doi: 10.1097/MCO.0000000000000089.
[46] Josic D, Giacometti J. Foodomics-Use of integrated omics in nutrition, food technology and biotechnology. Journal Data Mining Genomics Proteomics 2013; 4: 106. doi: 10.4172/2153-0602.1000e106.
[47] Reid LM, O'Donnel CP, Downey G. Recent technological advances for the determination of food authenticity. Trends in Food Science and Technology. 2006; 17: 344-353.
[48] Gašo-Sokač D, Kovač S, Josić D. Application of proteomics in food technology and food biotechnology : Process development, quality control and product safety. Food Technology and Biotechnology. 2010; 48: 284-295.
[49] Gacula MC. Descriptive sensory analysis in practice. Food and Nutrition Press, Trumbull, CT, 1997.
[50] Monteiro B, Vilela A, Correia E. Sensory profile of pink port wines: Development of a flavour lexicon. Flavour and Fragrance Journal. 2014; 29: 50-58.
[51] World Coffee Research. Sensory Lexicon. Unabridged Definition and References. First Edition, 2016, ISBN 978-0-9971542-0-7. 50 pages.
[52] Koppel K, Chambers E. Development and application of a lexicon to describe the flavor of pomegranate juice. Journal of Sensory Studies. 2010; 25: 819-837.
[53] Limpawattana M, Shewfelt RL. Flavor lexicon for sensory descriptive profiling of different rice types. Journal of Food Science. 2010; 75(4): S199-205. doi: 10.1111/j.1750-3841.2010.01577.x.
[54] Hongsoongnern P, Chambers E. A lexicon for texture and flavor characteristics of fresh and processed tomatoes. Journal of Sensory Studies. 2008; 23: 583-599.
[55] Vilela A, Monteiro B, Correia E. Sensory profile of Port wines: Categorical principal component analysis, an approach for sensory data treatment. Ciência e Técnica Vitivinícola. 2015; 30(1): 1-8.
[56] Murray JM, Delahunty CM, Baxter IA. Descriptive sensory analysis: past, present and future, Food Research International. 2001; 34, 6: 461-471. http://dx.doi.org/10.1016/S0963-9969(01)00070-9.
[57] Caul JF. The Profile method of flavor analysis. advances in food research. 1957; 7: 1-40. https://doi.org/10.1016/S0065-2628(08)60245-1.
[58] Lawless HT. Laboratory exercises for sensory evaluation. Flavor profile method. Food Science Text Series. 2012; 2: 79-84.
[59] Brandt MA, Skinner EZ, Coleman JA. Texture profile method. Journal of Food Science. 1963; 28: 404-409. doi:10.1111/j.1365-2621.1963.tb00218.x.
[60] Paula AM, Conti-Silva AC. Texture profile and correlation between sensory and instrumental analyses on extruded snacks, Journal of Food Engineering. 2014; 121: 9-14. http://dx.doi.org/10.1016/j.jfoodeng.2013.08.007.
[61] Stone H, Sidel JL, Oliver S, Woolsey A, Singleton RC. Sensory evaluation by quantitative descriptive analysis. Food Technology. 1974; 28(11): 24-33.
[62] Meilgaard MC, Civille GV, Carr BT. Sensory evaluation techniques. (2nd ed.), CRC Press, Boca Raton, FL, 1991.
[63] Yamagata Y, Sugawara Y. Sensory evaluation spectrum method as a descriptive sensory analysis. Psychology. 2014; 5: 1591-1610. http://dx.doi.org/10.4236/psych.2014.514170.
[64] Delarue J, Lawlor B, Rogeaux M. Rapid Sensory profiling techniques: Applications in new product development and consumer research, Elsevier, 2014.
[65] Langron SP. The application of procrustes statistics to sensory profiling A.A. Williams, R.K. Atkin (Eds.), Sensory quality in food and beverages: definition, measurement and control, Ellis Horwood Ltd, Chichester. 1983, pp. 89-95.
[66] Santos DAM, Lobo JST, Araújo LM, Deliza R, Marcellini PS. free choice profiling, acceptance and purchase intention In the evaluation of different biscuit formulations. Ciência e Agrotecnologia. 2015; 39(6): 613-623. https://dx.doi.org/10.1590/S1413-70542015000600008.
[67] Amerine MA, Pangborn RM, Roessler EB. Principals of sensory evaluation of food. Academic Press, New York, 1965.

[68] Brattoli M, Cisternino E, Dambruoso PR, de Gennaro G, Giungato P, Mazzone A, Palmisani J, Tutino M. Gas chromatography analysis with olfactometric detection (GC-O) as a useful methodology for chemical characterization of odorous compounds. Sensors (Basel, Switzerland). 2013; 13(12): 16759-16800. http://doi.org/10.3390/s131216759.
[69] iSens (Instrumental Sensometry research group), http://www.quimica.urv.cat/w3qaea/. Acessed 28 June 2017.
[70] Persaud KC, Dodd G. Analysis of discrimination mechanisms in the mammalian olfactory system using a model nose. Nature. 1982; 299: 352-355.
[71] Davide FAM, Di Natale C, D'Amico A. Self-organizing sensory maps in odour classification mimicking. Biosensors and Bioelectronics. 1995; 10: 203-218.
[72] Yea B, Konishi R, Osaki T, Sugahara K. The discrimination of many kinds of odor species using fuzzy reasoning and neural networks. Sensors and Actuators. 1994; 45: 159-165.
[73] Ouellette J. Electronic noses sniff our new markets. Industrial Physics. 1999; 5: 26-29.
[74] Pearce TC, Schiffman SS, Nagle HT, Gardner JW. Handbook of machine olfaction: electronic nose technology. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2003.
[75] Wilson AD, Baietto M. Applications and advances in electronic-nose technologies. Sensors (Basel, Switzerland), 2009; 9(7): 5099–5148. http://doi.org/10.3390/s90705099.
[76] Turner APF, Magan N. Electronic noses and disease diagnostics. Nature Reviews Microbiology. 2004; 2: 161-166. doi:10.1038/nrmicro823.
[77] Ghasemi-Varnamkhasti M, Mohtasebi SS, Siadat M, Balasubramanian S. Meat quality assessment by electronic nose (machine olfaction technology). Sensors. 2009; 9: 6058-6083.
[78] Ghasemi-Varnamkhasti M, Aghbashlo M. Electronic nose and electronic mucosa as innovative instruments for real-time monitoring of food dryers. Trends in Food Science and Technology. 2014; 38: 158-166.
[79] Kiani S, Minaei S, Ghasemi-Varnamkhasti M. Application of electronic nose systems for assessing quality of medicinal and aromatic plant products: A review, Journal of Applied Research on Medicinal and Aromatic Plants. 2016; 3(1): 1-9. http://dx.doi.org/10.1016/j.jarmap.2015.12.002.
[80] Polshin E, Rudnitskaya A, Kirsanov D, Legin A, Saison D, Delvaux F, Delvaux FR, Nicolaï BM, Lammertyn J. Electronic tongue as a screening tool for rapid analysis of beer. Talanta. 2010; 81: 88-94.
[81] Rudnitskaya A, Rocha SM, Legin A, Pereira V, Marques JC. Evaluation of the feasibility of the electronic tongue as a rapid analytical tool for wine age prediction and quantification of the organic acids and phenolic compounds. The case-study of Madeira wine. Analytical Chemical Acta. 2010; 662: 82-89.
[82] Riul A, Dantas CAR, Miyazakic CM, Oliveira ON. Recent advances in electronic tongues. The Analyst, Cambridge. 2010; 135(10): 2481-2495. http://www.producao.usp.br/handle/BDPI/50163.
[83] Baldwin EA, Bai J, Plotto A, Dea S. Electronic noses and tongues: Applications for the food and pharmaceutical industries. Sensors (Basel, Switzerland). 2011; 11(5): 4744-4766. http://doi.org/10.3390/s110504744.
[84] Parra V, Arrieta AA, Fernandez-Escudero JA, Garcia H, Apetrei C, Rodriguez-Mendez ML, Saja JA. E-tongue based on a hybrid array of voltammetric sensors based on phthalocyanines, perylene derivatives and conducting polymers: Discrimination capability towards red wines elaborated with different varieties of grapes. Sensors and Actuators B: Chemical. 2006; 115: 54-61.

Food education: an essential tool for stimulating households' cognition of nutritional values of safe and quality food consumption in Nigeria

Okanlade A. Lawal-Adebowale

Department of Agricultural Extension and Rural Development, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria

Food consumption, especially when nutritionally balanced, is crucial to survival and healthy living of individuals across the globe. As a result, accessibility and affordability of quality food by households becomes essential, particularly in a developing country like Nigeria, where food supply is inadvertently inadequate due to poor system of agricultural practices, postharvest handling and poor-resource status of most of the households in the country. This notwithstanding, efforts are made to ensuring availability of food resources of various kinds for households consumption. Commonly available crop-based food in the country include fruits, vegetables, grains, legumes, tubers, roots, and meat sources, namely fish, snails, poultry and beef, and other animal products. Consumption of these classes of foods are though processed or prepared into dishes of different kinds, attendant challenges in consumption of the available food resources include heavy dependent of most households on carbohydrate-laden foods with less consumption of protein and vitamin-rich foods, and poor quality of the food for safe consumption. The resultant effect of this is poor health condition often reflected in form of undernourishment and malnutrition. In children is a reflection of stunt growth and underweight. For the poor feeding condition to be alleviated, it becomes essential to have the households educated on nutritional values of food and pattern of consumption. Food education, which entails provision of technical information on nutritional content of specific food sources, is meant to give the needed insight to households on the specific kind of food or combination of foods to be consumed on daily and regular basis. Such educational provision, not only serves as enlightenment of the households on food nutrition, but empowers them to cognitively take the initiatives for food selection for safe consumption with a view to enhancing their health status.

Keywords: Food resources; food nutrients; food education; safe and quality food consumption; Nigeria

1. Introduction

Among the basic and essential needs of life, food outstandingly remains the prime of them all on the ground that it is crucial, not only to man's survival but his quality living. The value of food is obviously reflected in its necessity **for growth and maintenance of the body; it provides the body with** needed calories for energy building and strength development for body fitness.Food provides essential nutrients to keep the body healthy and as well enhances the physiological functioning of the body in terms of maintenance of the heartbeat, homeostasis, and brain and organ activities[1]. Derivation of energy from food is brought about by breakdown of the food molecule in the process of digestion. The energy, which is then converted to glucose, fuels the operation of the body cells and organs as a system. Emphasising the importance of food, it was stressed that nutritional value of food cannot be replicated by nutritional supplements on the ground that proper foods provide calcium to build and strengthen bones, protein and iron for muscle maintenance and other nutrients that allow the optimal functioning of the body's organs. In view of this submission, it suffices to say that, food should not just be adequately available to mankind at all times and at affordable rate, but should be nutritionally-rich for a guaranteed healthy and quality living.

Agriculture however remains the main source food for mankind and this may be gotten directly from farms or markets in the raw form and in the processed form from open markets and stores. In this wise, man's efforts had largely been directed at ensuring sustainable food production, widespread distribution, accessibility and affordability for regular consumption and derivation of physiological, psychological and social benefits. To boost agricultural production, investment in agricultural research and development has been on the increase with a view to developing new or improved technologies and practices for farmers' adoption and adaptation in their farming environment. Alongside research and development in agriculture is dissemination and sharing of agro-knowledge on production technologies and innovative practices among the farmers, and development of production capacity of the farmers.The cumulative result of agricultural research is increased agricultural productivity [2]. On this account, the whole world is no longer short of food as agricultural development has been able to respond to the rising demand for both crop and animal-based foods [3].

2. Food situation in Nigeria

Nigeria though runs an oil-based economy; agriculture equally plays significant roles in the nation's economy accounting for 40% of the nation's Gross Domestic Product (GDP) and provides employment, both formal and informal, for about 60% of the Nigerian population. Commonly produced crops and farm animals are however

determined by the agro-ecological features of the different regions of the country. With farm enterprise production across the country, the nation has the potentials for feeding of its teeming population, producing as much as 6.7milliom tons of rice; 70, 000 tons of wheat; 10.7million tons of maize; and 679, 000 tons of soybean; and 50million tons of cassava as at 2014, largely for both home consumption and to some extent [4] [5]. Alongside crop production is livestock production in the country where farm animals such as cattle, sheep, goats, poultry, pigs and fish are largely reared for home consumption. The rural environment though constitutes the main base of agricultural production in Nigeria; field observation shows that agricultural practice is fast growing in urban environment, with the average to low income earning households cultivating food crop of choice and farm animals at subsistent level for meat, milk and egg production in the country.

Given the agricultural production efforts in Nigeria, it is unfortunate that the mode and status of agricultural production in the country has not really transform the country into a food secure nation; and as such the home-based food supply system has not kept pace with the growing population. In the light of this, Nigeria is said to be food insufficient given that the nation's average growth rate of food demand is 5% per annum against the annual growth rate of food supply at 3%. In addition, about 5% Nigerians are food secure, 65% are semi-food secure and 30% are food insecure. In addition to farming for food production in Nigeria, the country had equally resorted to food importation with a view to meeting the food demand of its teeming population [6].

Notwithstanding the state of Nigerian agriculture and production system, the nation has resorted to food importation as alternative to taking care of shortfall in home production. Nigeria spent about US$11billion (₦3.1trillion) on importations of four major food sources, which are rice, wheat, sugar and fish annually [7]. In line with this, the country is known to have spend a whopping $2billion dollars importing about six million tons of wheat, $750 million on rice $700 million on sugar and $500 million on milk and other dairy products [8]. As at 2013, Nigeria's food imports, was 2.187million metric tons of rice, 4.356million metric tons of wheat, 1,200 metric tons of maize and 12, 757metric tons of soybean [9]. Reflecting on the estimates of food importations in 2010, Nigeria spent as much as US$635 billion on wheat, US$356 billion on rice, US$217 billion on sugar and US$97 billion on fish as at 2010 [10]– a trend that has not abated till date due to poor state of the country's agriculture.

Nigeria's dependent on food importations may though be of great implications for the country's economy, particularly affecting the average to low-income earning households in the face of global rise in food prices; the resultant effect of the importations, alongside the home food production is sustained food supply system in the country; which has thus bailed the nation out of the prospect of large scale famine. In the light of this, Nigeria is though food deficient. it has not suffered any major catastrophe that could precipitate scourges of famine, mass hunger and food crisis [8].

3. Food types and their nutritional values in Nigeria

Given that efforts are ever geared toward improved agricultural production in Nigeria, and coupled with food importation in the country, it suggests that food production and distribution remained sustained in the country. In other words, every inhabitants of the country could in one way or the other have access to food for consumption. Such foods, which are available in various kinds, could be sourced either directly from farms or purchase in the open markets. The commonly available food in the country thus cut across the six classes of food groups, namely carbohydrates, protein, fats and oil, mineral, vitamins and water. In the light of this are the following commonly available food types and categories in Nigeria:

- **Tuber crops:** cassava, yam, potatoes and cocoyam (taro)
- **Cereals:** maize, rice, wheat, millet and guinea corn
- **Legumes:** cowpea, soybean and groundnuts
- **Fruits:** banana, mango, cherry, pine apple, almond, pawpaw, watermelon, citrus, apple, carrots, sugar cane, garden egg, cucumber
- **Vegetables:** amaranths, waterleaves, pumpkin leaves, cabbage, jute mallow, okra, *Ogbono* (African bush mango)
- **Oil-based foods:** palm oil, groundnut oil and melon oil
- **Spices:** pepper, onions, tomatoes, *iru* (Parkia biglobosa)
- **Meat sources:** cattle, sheep, goats, poultry, pigs and fish
- **Animal products:** meat, milk and eggs
- **Other common food resources:** Bread, macaroni/pasta, semovita, yoghourt, fruit juice

The food resources are however processed into different dishes for consumption. For instance, cassava may though be consumed as tuber when boiled; it is largely processed into local food stuffs such as *gari* (granular in texture)*, elubo lafun* (powdery in texture)*, fufu* (paste or powdery in texture) and tapioca which are then process into consumable dishes such as *eba, amala, fufu* and tapioca pap. These dishes constitute staple foods in Nigeria, particularly in southern region of the country, where cassava is widely cultivated and locally consumed. These cassava-based foods are however often consumed with stew of various kinds. On a similar note, yam is consumed when boiled, fried, roasted or prepared as porridge. Boiled yam is consumed with either palm or groundnut oil or fried egg. It could as well be pounded or

prepared; and where yam has been processed into yam flour, locally referred to *elubo isu,* it is prepared as *amala,* particularly among the Yoruba tribe in the country. Pounded yam and *amala* are thus consumed with stew of various kinds as may be desired by individual or households. While *amala* may be common to southwest Nigeria, *eba/gari and fufu* are common to both southwest and southeast regions of the country. Cocoyam is equally common to both regions where it is consumed boiled, fried or pounded. Potato is however common to all regions of the country where it is largely consumed in boiled or fried forms. Among all the available food resources for consumption in Nigeria, cassava and cassava-based foods are cheapest food resource and serves as hunger-relieving food.

Following tuber crops is wide consumption of cereal crops such as maize, rice, wheat, millet and guinea corn across the country. Among these grains, rice stands out as the most widely consumed of them all. It constitutes a major staple that alternates between tuber crop-based foods on daily basis. Rice is most prepared and consumed as white rice, jollof rice, fried and coconut rice. The white rice is mostly taken with pepper stew and sometimes with fried plantain, locally referred to as *dodo* in southwest Nigeria. In northern part of the country, white rice is pounded to form *tuwon shinkafa*, which is taken with a bit of powdery pepper and groundnut oil. In southwest and southeast regions, rice, to a lesser extent, is grinded into powdery form – grind rice, such that its preparation for consumption takes the same form as *amala or eba* and thereof taken with stew of choice. Maize on the other hand is mostly consumed as boiled maize around the country; and sometimes allowed to dry and grinded to powdery form for preparation of pastry food locally referred to *tuwo* in southwest Nigeria and *tuwon masara* in northern region. Other food sources from maize when fermented include *eko and ogi (pap),* but these are less taken as part of normal meal. Wheat is mostly taken in different forms which are wheat pap – when fermented, and wheat *amala* – a solid food similar to *amala* made from cassava and yam powder and consumed with stew of choice. Millets and guinea corn or sorghum are mostly processed into pap, after fermentation, in southern part of Nigeria while they are processed into *fura,* often consumed with local milk commonly known as *nono,* largely in the northern region of the country or among the Hausa tribe.

Among leguminous crops, cowpea stands out as the most widely consumed across Nigeria. It is prepared by boiling either with a mixture of pepper and palm oil or without any mixture in which case fried pepper stew is prepared to garnish the beans for consumption. Bean preparation may also be in mixture of plantain or yam thereby forming a resemblance of porridge. On another note, cowpea may be prepared as stew often referred to as *gebgiri*, among the Yoruba tribe in Nigeria. Such bean stew is often used to spice *amala* for consumption, mostly in southwest region of Nigeria. In addition to this is processing of cowpea into other food resources such as *moinmoin, ofuloju and akara* when grinded into paste form. While m*oinmoin* and *akara* are often consumed with pap and *eko, moinmoin* may be consumed with rice and bread. Groundnuts, whether boiled or roasted, are often taken as refreshment rather foods in Nigeria, hardly is soybean consumed as food except for consumption as soymilk. In northern part of the country, groundnuts, on extraction of oil (groundnut oil) is processed into groundnut cake, locally referred to as *kuli or kulikuli,* which is as well consumed as refreshment.

Vegetables, oils, spices, meat and fish are other essential food resources of value in the Nigeria's context. These food resources serve as component of most of the staple foods consumed by most households in Nigeria. For instance, staple foods such as *eba, amala, fufu,* semovita and pounded yam are consumed with stew mostly prepared from vegetables and pepper. Vegetables, which may be amaranths, waterleaves, pumpkin leaves, is on the other hand prepared as stew by adding vegetable of choice to the same mixture of condiment as used for pepper stew. This may however be garnished with dry fish and/or crayfish, *iru* and other seasonings. In place of preparation of vegetable as the main stew, it is prepared in small quantity with a view to serving it alongside pepper stew. Alternative to vegetable stew as support to pepper stew is jute mallow, okra or *Ogbon,* which are commonly referred to as *draw soup* due to their caustic or elastic nature. Stew is mostly prepared at interval of three to four days and kept fresh and tasty for the days it may last. With regular availability of stew at home, it becomes possible for households to have something to eat on daily basis as this is essential to consuming most food resources in the country.

Fruits such as banana, mango, cherry, pine apple, almond, pawpaw, watermelon, citrus, apple, carrots, sugar cane, and garden egg are readily available across Nigeria. Although, fruits do not constitute staple food in Nigeria, they serve as supplements to the main staple foods and are consumed as may be available and affordable among the Nigerian households. Fruits are however seasonally available in the country, with some kind of fruits available at a particular period of the year. In essence, all through the year, at least one or two kinds of fruits will certainly be available for consumption by Nigerians. In addition to availability of raw fruits across the country is availability of fruit juice either produced by home industries such as FUMAN, Chi Industry, Nigerian Bottling Company, or imported from other countries. Such fruit juice comes in various brands and sizes and are widely available both in the open and super markets across the country. On another note is availability of milk and milk produces such yoghurt, canned milk, fan ice, powdered milk, which of course are mainly produced by milk industries. Yoghourt and fruit juice are however consumed to spice up the staple food or at any other time as refreshment. This of course is largely common among the affluent ones and those with high paying jobs as result of high cost of acquiring these food resources.

Other food resources of high value in Nigeria are bread, macaroni/pasta, noodles and *semovita* which are largely industrial-based foods. These set of foods, which are more common among the urban dwellers, are gotten from open markets or supermarkets in the country. Bread, which is outstandingly common and regularly consumed by households across the country, even in the rural areas, is a product of baking industries produced from wheat flour, and more

recently from High Quality Cassava Flour, and mixture of other baking ingredients. Based on high demand, bread from fine flour is commonly available in the country; while the whole wheat bread, which is specially demanded for by those who value the product, is less available in the country. Macaroni/pasta and noodles of various brands are made by large food industries in the country and are widely available for purchase and consumption. These set of foods are though quickly prepared in a lesser time, their consumption depends on both tastes and economic status of households as they are relatively expensive in comparison with other non-industrial staple food resources which are mostly gotten either directly from the farm or in the open markets for home processing and preparation for consumption.

The highlighted food resources in Nigeria suggest that available food types in the country are rich in different nutritive values. For instance tuber crops are known to be energy-giving foods, rich in carbohydrate but low in protein content. Root and tuber crops are low in sulphur-containing amino acids and also deficient in most vitamins and minerals. Tuber crops such as cassava, potato and yam are however rich in vitamin C with the yellow varieties of sweet potato, yam and cassava rich in carotene or pro-vitamin A and significant amount of dietary fibre [11.12]. In general, cassava provides 160 calorie per 100g with about 69% sucrose and 16-17% amylase. In the same vein, yam, which is made up of 72.5% water, contains 16.8% crude fibre, 9.02% carbohydrate, 6.8% iron, 2.06% Ash and 0.83% protein. Given the nutritional value of yam, more than average energy requirement for human adult could be gotten from yam as consumption of 100gm of the tuber gives 93.6% calorie/kg of energy; and can as well sustain the daily iron requirement of all age group with provision of 6gl/100gm [13]. *Eba,* a heavy food resources made from cassava and often referred to as swallow, is 99% carbohydrate and provide about 360 calorie [14]; pounded yam provides 400 calorie, *amala isu* (made from yam flour) provides 250 calorie, 2% vitamin A and 70% iron; and *amala lafun* (made from cassava flour), as highlighted by has 1.2% protein, 1.6% crude fibre, 0.4% fat, 0.8% ash and 13% moisture [15].

Cereals or grains related food resources such as rice, maize, wheat, millets and guinea corn/sorghum are equally rich in carbohydrate and which is approximately 75% of their nutritional value. Cereals are major sources of carbohydrate, protein, B vitamins and minerals for the world's population [16]. The protein content ranged between 6–15% [17]; and are important sources of most B vitamins, especially thiamine, riboflavin and niacin and appreciable amounts of vitamin E [18]. Cereal-based foods are equally rich in minerals such as potassium and wholegrain cereals contain considerable amounts of iron, magnesium and zinc, as well as lower levels of trace elements such as selenium. Rice contains the highest level of selenium among the cereal grains, providing between 10 and 13µg per 100g [15]. Furthermore, about 28.7g and provides130 calorie per 100g; it has protein and fat contents of about 2.4g and 0.2g respectively. It however lacks most vitamins but contain 1% calcium. Maize or corn 60-70% starch and vitamins B group, D, E, K and pro-vitamin A [18]. Corn is also rich source of minerals such as potassium, sodium, calcium, magnesium, iron, copper, manganese, phosphorus, selenium, zinc and iodine. While cereals generally lack vitamin A, yellow corn is rich in yellow corn, no beta carotene.

Wheat contains a lot of starch or carbohydrate, 11% of protein, 2% of fat, 13% of fibre, 1% of mineral (iron, phosphorus, potassium, magnesium, calcium, zinc, manganese) and also high amount of vitamins of B group and vitamin PP. Wheat germs are rich in vitamin E and enzymes. Millet though contains about 63.2% starch, it is a good source of protein, containing about 13.6% crude protein, and essential amino acids, with exception of lysine and threonine, [19, 20]. The grains also contain 3.6 to 4.8% fat, vitamins B_3, A, PP, as well as mineral salts such as magnesium, potassium, phosphorus, silicon, iron and copper; but do not contain gluten. Sorghum nutritional contents consist of 70% of carbohydrates (mainly starch), and 18% protein, with significant share of lysine and tryptophan, about 5% fat, vitamins (mainly groups B), minerals, especially magnesium, calcium and iron. However, cereals lack some vitamins such as vitamin C, vitamin B12, vitamin A and, apart from yellow corn, no bet-carotene; and also low in mineral like sodium.

Leguminous-based food resources are on the other hand rich in protein, fibre, iron and potassium but low in calorie and fat. Legumes contain complex carbohydrates, protein with a good amino acid profile, important vitamins – B vitamins, folates, ascorbic acid and tocopherols, minerals antioxidants, polyphenols and numerous other phytochemicals with useful biological activities [21, 22]. In addition, legumes are good sources of water-soluble vitamins, particularly thiamine (*Vitamin B1*), riboflavin (*Vitamin B2*), niacin (*Vitamin B3*), pyridoxine (*Vitamin B6*), foliate and excellent sources of minerals such as calcium, copper, iron, magnesium, phosphorus, potassium and zinc. In addition to high protein content of legumes, leguminous crops such as beans and cowpea (pulses) store high level of carbohydrate in their dry seed but low lipid or fat content; while soybean and groundnuts (oilseeds) store high lipid content but low carbohydrate. Legumes in general are however poor sources of fat-soluble vitamins and vitamin C; and also low in sodium minerals.

Most food from roots and tubers are often consumed with stew in Nigeria with such stew made from pepper, vegetables and spices. Stew sources such as vegetables, specifically amaranth, pepper, jute and cabbage, are highly rich in vitamins particularly vitamin A and C [23]. In addition, amaranth, jute and cabbage were indicated to be rich in iron and calcium. In addition, all green, yellow and orange vegetables are rich in vitamins and minerals such as calcium, magnesium, potassium, iron, beta-carotene, vitamin B-complex, C, A and K. Melon (*egusi*) soup enriched with stockfish and meat provides about 700 calorie and about 60% fat. Stew, be it pepper or vegetable, are often prepared with vegetable fruits or spices such as red pepper, usually in form of chilli, scotch bonnet and bell types of pepper; onions and tomatoes. These are known to be rich in vitamins C, A, B_6 and magnesium. Onions however lack Vitamin

A. Palm oil and/or groundnut oil, with which stew is generally prepared, are largely rich in fat and have high calorie content. Both types of oils are however devoid of vitamins and minerals. In addition to the use for stew preparation is the use of the oils for frying food resources such as meat, fish, egg, yam, plantain, *akara* (bean cake); and mixed with bean past for production of *moinmoin*.

Alongside the nutritive value of vegetable or pepper-based stew is the nutritive value of fresh fruits. Fruits are are rich of simple sugar, fibre, enzymes, minerals and vitamins, particularly vitamin C, calcium and phosphorous. They are also energy-giving foods but have low carbohydrate, protein, fat and fibre contents [24]. Nutritive value of fruits however varies from fruit to fruit. Given the nutritive values of different fruits, guava and citrus are rich sources of vitamin C at 299 and 63-68mg/100g respectively, while banana, cashew, apple and almond are rich sources of vitamin B_1 at 150, 230, 120, 240mg/100g respectively [25]. **Riboflavin or vitamin B_2 is readily found in p**apaya (200mg/100g) and pineapple (120mg/100g). Vitamin A could be readily derived from mango (4800 i.e., per 100 g) and papaya (2020 i.e. per 100g).

Food resources such as bread, macaroni/pasta, noodles and *semovita* are equally energy-giving foods but are rich in some nutrients given the crop produce from which they are produced. About 40% of bread is carbohydrate, foliate (25μg/100g), calcium (177mg/100g) and 8–9% is protein but is low in fat (less than 3 g of fat/100 g) [15]. Fibre content of bread is however significantly higher in wholemeal and brown bread than white bread. Pasta, which is traditionally made from very hard (durum) wheat, though contains about 22% carbohydrate; it is high in protein content at about 3g/100g and calcium (7mg/100g). It is however low in fat, thiamine and iron. Animal food resources, such as meat, fish, milk and milk product are highly rich in protein, calcium.

4. Food accessibility and consumption pattern in Nigeria

Food is known to be crucial to life and as such its need for consumption cannot be taken for granted. Emphasis in this regard is underscored by a dictum in Nigeria that says, a food secured person never lies in poverty. Consequently, humans in general make frantic effort to ensure that food is regularly and adequately available for consumption. In Nigeria, food is generally gotten in two main ways; which could either be by direct harvest from personal farms and by purchase from markets or farm gates. Farm production, be it in rural or urban areas, provided the farming households direct sources of food for home consumption and where possible, have the excess for marketing. This creates an opportunity for the non-farming households to have access to food by purchase. Food purchase from the markets could either be in raw form which is then processed or prepared at home for household consumption or from street food vendors and restaurants or fast food restaurants. While the street food vendors are mostly patronised by average to low income earners, restaurants and fast foods centers are largely patronised by high income earners. In addition to farming for food production in Nigeria, the country had equally resorted to food importation with a view to meeting the food demand of its teeming population. In view of farm cultivation and massive food importation, nationwide distribution of the available foods through the local marketing structure has made food of various kinds available in the country for purchase and consumption by individuals and households at any point in time.

The extent to which food becomes accessible to households in Nigeria however depends on their production resources and/or their economic status. In views of this, socioeconomic status of individuals or households constitutes a major determinant of their level of food security [26]. Consequently, the poor-resource farmers largely produce at subsistence level for home consumption, with less quantity available for sales, while the highly resourced-farmers produce on commercial scale and as such consume from the available surplus. Economic implication of this is that, those that consume the larger proportion of food produced than what is available for marketing are usually left with the least disposable income to cater for other basic needs of life [27]. In the same vein, expenses on food by households cost as much as 60 – 70% of the annual income of most them. This situation accounted for the increased state of poverty in country. In attempt to wriggle out of poverty condition, most households, particularly the low to average income earners in the country, have resorted to reducing expenditure on food whereby they either consume less expensive food resources, which are mostly carbohydrate-laden or skip at least one meal per day. This goes in line with the submission that limited income causes people to restrict the number, quantity and quality of meals they consume, reduce the dietary variety, and look for inexpensively processed food resources [28].

In view of this, a survey on rural households' food structures shows that they consumed ₦3, 465.13 worth of carbohydrate foods; ₦750.54 of proteins and ₦191.43 of vitamins; and on the average consumes ₦1469 worth of food per month [27]. The authors however stressed that the consumed food resources were in the short falls of 18% carbohydrate and 11% protein intake over three years. A similar study on food consumption pattern among adolescent in selected southwest Nigerian secondary schools reveals a higher daily energy intake among 66% of the adolescents and higher carbohydrate intake among 62% of them against the lower intake of fat and protein among 51% and 42% of the adolescents, respectively but low iron intake [29]. In addition to this is skipping of breakfast with less consumption of fruits and vegetables; and milk and milk products. In line with this is the submission that most Nigerian households lack nutrition-oriented food for consumption as their meals are largely characterized by low intakes of protein, energy, iron, calcium, zinc, thiamin, and riboflavin in almost all age groups and in both sexes [30]. In view of this state of food consumption as observed among the Nigerian households, it suffices to say that food consumption by most households

is underscored by the need to satisfy hunger rather than the need to meet the body's nutrition requirement for healthy living. This chosen food consumption pattern accounted for widespread of malnutrition among the nation's citizens, particularly among the children under age five.

5. Health implication of food consumption pattern in Nigeria

Food consumption, as often reflected by individuals and households, is not necessarily meant for satisfaction of hunger, but for nourishment and enrichment of body's physiological and psychological functions. Adequate consumption of safe and quality food not only reduce the burden of non-communicable diseases but enhances and ensure physical growth and development of the body physiology, muscles and tissue repair, cell growth, healthy brain and organs. It also enhances strong bones and teeth, good eyesight, gives energy, prevents and fights off body disease. Poor feeding pattern however results in malnutrition underlined by either outright lack of or inadequate consumption of essential nutrients required for proper functioning and healthy maintenance of the body. Malnutrition, largely characterised by protein–energy malnutrition and micronutrient deficiencies, continues a major health burden in developing countries where hundreds of millions of pregnant women and young children particularly affected [31]. Evidence of short-term and long-term consequences of malnutrition include increased risk of morbidity, mortality and proneness to infectious diseases; hindrances of good physiological development and impaired cognitive or behavioural development in children [32, 33]; and in adulthood are reduced educational and productive capacity, and ill-health [34]. Other health related matters reflected by adults as a result of poor feeding pattern include obesity and diet-related diseases such as diabetes, hypertension or high blood pressure and heart disease [35].

On another note, marasmus and kwashiorkor, constitute the major manifestation of protein–energy malnutrition in children whereby the affected children are underweight, stunted and wasting [31]. With deviation from normal weight and height in relation to age as the standard measurement of protein–energy malnutrition in children – ie weight for age (underweight), height for age (stunting) and weight for height (wasting) [36], were able to ascertain that about 31% of all children in developing countries are underweight, 38% have stunted growth and 9% show wasting. As further stressed by the authors, the malnutrition may be severe in some children and this typified by wasting, edema or both. Most children with severe protein–energy malnutrition have asymptomatic infections because their immune system fails to respond with chemotaxis, opsonization and phagocytosis [31]. Severe protein–energy malnutrition also causes fatty degeneration of diverse organs, particularly the liver and heart; and chronic hypovolemia, which leads to secondary hyperaldosteronism. This status of malnutrition is usually manifested early in children between 6 months and 2 years of age as a result of early weaning, delayed introduction of complementary foods, a low-protein diet and severe or frequent infections [37, 38].

In addition to the health implications of the protein-energy malnutrition, poor dietary intake is known to result in micronutrient deficiency in most households around the world, particularly in the developing the countries. Such micronutrients include iron, iodine, vitamin A and zinc [31]. Iron is crucial to blood cell formation in the body and its deficiency thus results in anaemia – an ill-health situation that is characterised by deficient red blood cells or haemoglobin, or both [39]. This health condition becomes manifested in the wake of excessive bleeding, inadequate production of red blood cells, or excessive destruction of red blood cells. Iron deficiency, which rated as the most prevalent micronutrient deficiency around the world affects about 2billion people globally with about 70% of such ones in the developing countries. Furthermore, the underlying cause of poor iron intake, particularly in the right quantity and quality, is poverty status of most households. Arising from poor dietary and iron intake, about half of the pre-school children in developing countries are anaemic.

Vitamin A deficiency, which though impairs clear eyesight and proper functioning of the immune system, also contributes to anaemia by immobilizing iron in the reticuloendothelial system thereby reducing hemopoiesis and increasing susceptibility to infections [40]. On another note, diarrhea and related mortality has clearly been shown to be associated with vitamin A deficiency [41, 42]. Lack of iodine reduces the production of thyroid hormone and increases that of thyroid-stimulating hormone [43, 36]. In view of this, the thyroid gland becomes hyperplastic and goitrous leading to hypothyroidism develops. Zinc deficiency interferes with a variety of biological functions, such as gene expression, protein synthesis, skeletal growth, gonad development, appetite and immunity [44, 38]. Deficiency of this element is also a major determinant for diarrhoea and pneumonia.

Based on the foregoing, both children and adults in Nigeria are experiencing ill-health or abnormal health condition as a result of poor feeding pattern and inadequate micronutrient consumption. Effects of malnutrition though vary from city to city and between urban and rural areas, the degree of variation is found to be correlated with socioeconomic status of the households in Nigeria [45]. With regional and social disparities in Nigeria, malnutrition is prevalent in the northeast and northwest and much more among the poorest quintile [46]. In view of the criteria for the measure of protein-energy malnutrition, the observed status of malnutrition among the Nigerian households ranged between stunted (low height-for-age) and undernourished (low weight-for-age). In the same vein, about 11 million children under the age of five are stunted; and was further stressed that stunting happens when a child's brain and body do not get the right kind of food or nutrients in their first 1,000 days of life. With about half the children aged 6 to 59 months not receiving vitamin A supplementation, deficiency of this nutrient results in child growing up with lower immunity, and this readily

triggers frequent health problems and poor growth. In view of the correlation of malnutrition to child mortality is an indication that about 52% of child's death in Nigeria is attributable to malnutrition and 80% of the nation's children experienced moderate-to-mild malnutrition rather than severe malnutrition [47].

6. Food education practice in Nigeria

It is a known fact that food is crucial to both physiological and psychological growth of man; and enhances proper functioning of individuals' physiological make up and good health status. These functional roles of food are certainly accomplished only when man is placed on adequate and quality foods. In this wise, food consumption needs to be rich in all nutrients for real benefit to the body. It is however certain that a single set of food resources never contain all the required nutrients – water, carbohydrate, protein, fat, minerals and vitamins; but variety of food resources. In addition to quality food consumption is the need for safety of the food for safe consumption. For individuals or households to be conscious of the need for quality and safe food consumption, it becomes essential to have them educated on nutritional values of different food resources and proper handling of the food for safe and quality consumption. Where such food education is not in place, individuals or households become tilted toward consumption of junk or unhealthy foods. The rising case of epidemic of childhood obesity in the United States is due to consumption of non-healthy foods and to correctly solve this nutritional problem is the need for nutrition education.

Nutrition education is any type of actions designed to change knowledge, attitudes and behaviours of individuals, groups of individuals or populations to contribute to the prevention and control of malnutrition in all its forms, and any erroneous food consumption, including of course the economic aspect [48]. It as a set of planned educational activities targeted at certain population groups and aimed at acquiring healthy nutrition behaviours [49]. On a similar note is conceptualisation of nutrition education as any combination of educational strategies designed to facilitate voluntary adoption of food choices and other food- and nutrition related behaviours conducive to health and well-being [50]. American Dietetic Association – ADA conceived nutrition education as instruction or training intended to lead to acquisition of nutrition-related knowledge and/or skills and be provided in individual [51]. A common trend across these definitions is that nutrition education has to do with influencing the food consumption behaviour of certain group of people through instructional education. In essence, food or nutrition education may be described as conscious or deliberate efforts to provide certain set of people with technical information on variety of nutritious food resources and safe handling of the food with a view to influencing their food consumption behaviour toward becoming mindful of quality and safety of food types for consumption. The main goal of nutrition education is to make people aware of what constitutes a healthy diet and understand ways to improve their diets and their lifestyles [52]. The resultant effects of this is that, nutrition education helps individuals, families, and communities make informed choices about food and lifestyles that support their physiological health, economic, and social well-being.

Nutrition education generally takes place in schools, targeting young children basically because food habits in early stages of life is believed to determine practices and preferences in adulthood [52]. This is a similar experience in Nigeria where nutrition education takes place in schools for the purpose of educating school pupils and students on nutritious food and good eating habits. Teaching nutrition to children throughout their educational experience is a key to developing healthy eating habits [53]. Consequently, food nutrition takes place in Nigerian schools, though as part of health education curriculum for pupils in primary schools and part of home economics, health science and biology in secondary schools, for the purpose of influencing their eating behaviours toward safe and healthy food consumption. In higher schools, nutrition education is thought as course of discipline for development of professional food and nutrition practitioners. As reflected by the West African Examination Council's Syllabus for secondary school students (www.myschoolgist.com.ng), the nutrition education in Nigerian Secondary Schools aims at ensuring that the students acquire basic knowledge about food and nutrition; understand the relationship between nutrition and health; develop the ability to apply the general principles underlying meal planning, selection, preparation and serving of food to feed family and other consumers for different occasions; acquire research skills and use the information to experiment, develop and improve local dishes. But the fact that home environment extremely influences a child's eating behaviours [53] emphasises the need for extension of nutrition education beyond the classroom to the home environment using multiple channels of communication in the educational delivery. As further stressed by the author, integration of teachers, foodservice professionals, and family members in nutrition education strategies plays important roles in promoting life-long healthy eating habits. Consequently, schools need to reach out to parents with nutrition education with a view to shaping the parents' food consumption behaviours and reinforce the thought lessons on food nutrition in the school learners for healthy living of all.

Although, teachers and food nutrition professionals engaged pupils and students on food and nutrition education either in school environment, organised food conferences/workshop or hospitals, the practice is hardly linked with the home environment in Nigeria. That is, no conscious or deliberate nutrition education effort is put in place for education of homes and parents, but largely in formal school setting. In view of this, individuals and households in Nigeria have largely relied on their age-long food resources types and consumption pattern, as may have been inherited from parents or dictated by culture, to guide their food nutrition practise. In addition to this is informal nutrition education practice whereby people generally share information on food nutrition with one another either by physical contact or through the

internet and social media. In this wise, people are taking to different ways of improving their nutrition by exploring available food resources in the country to improve the nutritional status and healthy living. Instances in this regard are online information on food constituents, food resources and their characteristic nutrients, food combination for consumption, meal time, meals for children and other age groups. While some individuals or households with higher level of education have had taken it upon themselves to explore available nutrition information from the Internet or social media, and in some cases from radio and/or television broadcast as may be presented by resource persons on food and nutrition, to guide their food consumption and eating habits; it is extremely difficult for the less educated ones to do so, particularly for those in rural areas on account of outright absence of or poor development of basic infrastructure or communication facilities for information sourcing. This accounted for why cases of malnutrition or poor feeding pattern are higher in the Nigerian rural areas than its urban counterpart.

On another note is the value of nutrition education on food safety for safe consumption. This is underlined by the fact that nutritionally rich food does imply safe food as nutritionally rich food may not be safe for consumption. In this wise, part of the Nigerian education curriculum for students in schools centres on safe handling of food items particularly during preparation. The consulted West African Examination Council's Syllabus reveals objectives of the safe food education to include ability to apply the general principles underlying meal planning, selection, preparation and serving of food to feed family and other consumers for different occasions; understand the need for planning an efficient and safe kitchen; choose, use, care and store kitchen equipment and tools effectively; appreciate the importance of sanitation in the kitchen food preparation and service; apply basic principles underlying food processing, storage and preservation; acquire basic knowledge in consumer education; and acquire research skills and use the information to experiment, develop and improve local dishes. Education on safe consumption thus exposes the in-school learners to developing personal and kitchen hygiene in terms of general cleaning, waste disposal, pests and pest /control, as well as food hygiene in terms of food handling, identification and guard food borne diseases and food sanitation laws.

In similar way to educational dictates of food nutrition, home and environment hygiene is culturally dictated in Nigeria with the dictums: *hygienic practises override illnesses; cleanliness is next to godliness.* In this wise, Nigerian households and individuals are highly conscious of the need for maintaining hygienic environment and food consumption; and these accounted for less cases of food borne diseases in the country. Occasionally is information from the food regulatory agency in Nigeria – National Agency for Food, Drug and Administration Control (NAFDAC), on unhygienic and unsafe food resources that might be in circulation in the country for guidance of consumers and prevention of food-related diseases. This notwithstanding, it is obvious that most of the less educated and poor-resource households are less concerned with cosmetic appearance of food resources, particularly fruits, as they readily consumed dented or spotted fruits whether washed or unwashed; consumed dead animals on the ground that intensive cooking destroys germs and pathogens in foods. This suggests the need for further and strengthened food and nutrition education in the country.

7. Implications of food education for dietary behaviours of the Nigerian households

Given the role of food and nutrition education in stimulation of healthy eating behaviours and prevention and control of malnutrition in human society, its effective implementation potentially enhances improved dietary practices and motivate participants' change of dietary behaviours using the acquired knowledge and skills on how to make healthy food choices in the context of their lifestyles and economic resources (Food and Nutrition Service, 2010). In view of the mode of food education practice in Nigeria, field observation shows that individuals and households in the country have been exploring such information for improving their nutritional intake. For instance, many of the pregnant women and nursing mothers, who are mostly educated on food nutrition during the antenatal period in hospitals and health centres in the country, have taken informed decisions to ensure adequate and quality feeding during pregnancy and to the practice of exclusive breast feeding for the first six months of birth as way to nourish the child for healthy growth and psychological development; and thereafter ensure that the infants are placed on quality foods.

On weaning, the wealthy households may able to afford canned baby foods, the poor-resourced ones often resort to cheap and less nutritionally rich food for their feeding their babies, particularly pap (*ogi*) – fermented corn as meal. Realisation of this fact has brought about education of the poor-resourced households on fortified pap such that a number of grains, namely, wheat, guinea corn, millets and soybean are jointly fermented with maize and thereafter grinded to form a fortified pap or *ogi* for onward feeding of the infants. And if at all pap is to be solely made from a single type of grains it should rather be millets or guinea corn instead of maize due to high nutritional value the grain. This practise of fortified pap, which outstandingly recommended, is based on the fact that the included grains are high in protein content, which is essential to enhancing infant's growth and development.

Food consumption is a daily venture with individuals and households consuming between two and three meals per day. Of all the daily meals, field observation reveals breakfast as the most crucial given that the consumed morning food replaces the lost energy overnight as a result 'fasting in sleep' thereby re-energising the body and the brain for the day's activities. For the school-age children is also the provision of breakfast as a way to strengthen their health and ensure concentration during school learning. Although the quality and quantity of food given to a child or consumed by households is largely determined by the economic status of the child's household, the impact of food education is that

most households have found it necessary to provide breakfast, as least for the kids, and in most cases for the adolescents, if at all the adults may not take breakfast.

Most foods consumed by households often undergo one form of processing or the other in the cause of preparation for household consumption. Such preparation process causes the loss or reduction of the amount of nutrients in food, particularly processes that expose foods to high levels of heat, light, and/or oxygen. In this wise, some of the Nigerian households have taken to careful boiling or frying of foods to prevent the loss of food nutrients. For instance, against the traditional practice of parboiling or washing vegetables with hot water before cooking is now the informed practice of washing vegetables with clean but cold water and slightly cooked as stew as a way prevent the loss or reduction of its nutrition contents. Foods to be preserved, particularly fruits and vegetables, are also known to be blanched by briefly boiling or steaming with a view to killing enzymes that would otherwise cause unwanted changes to the food color, flavor, texture and nutrient density during preservation and storage. Although the rate of nutrient loss from food storage and preservation is dramatically reduced by blanching, the heating practice equally causes some nutrient loss, particularly a reduction in water soluble nutrients [54]. On a similar note is the practice of less heating of cooking (palm and vegetable) oils against the age-long practice of overheating which nutrition education has revealed to breakdown quality fats into harmful trans fats and some oils do turn rancid at high temperature.

Against the backdrop of consumption of oily and fatty foods and heavy or carbohydrate-laden, which has in turn lead to obesity heaviness of the body, is the avoidance of such types of food for fruits and lighter foods for consumption. Field observation shows that the option of fruit and light food consumption is referred to *keto* – which simply means keep-to-fit. The nutrition guides have largely been drawn from online media and sharing among friends. The resultant effect of such dietary behaviour is significant loss of weight to a desired level by individuals who had undergone such dietary practice. The loss of weight is believed to be crucial to preventing cardiovascular disease which is often manifested as high blood pressure, high cholesterol, and Type 2 diabetes [55].

Water and drinks are also essential component of nutrition and dietary intake. On this account is the influence of educative information on clean and adequate water consumption for physiological and metabolic functioning of the body system. Interactions with some individual on water intake behaviour reveals that some have made it a habit to take one or two glasses of water just before going to bed and first thing on waken up in the morning. This is based on the information sufficient water in the body system lowers blood pressure, prevents heart attack, activate internal organ and aid digestion. With regards to drinks is avoidance too much juicy and very cold drinks in order to guard against lowering the blood temperature and prevents pneumonia.

Hygienic practice is crucial to ensuring safe food consumption. With food education on safe food handling for safe consumption is the informed choice of mindful of food handling by individuals and household in Nigeria. Interactive with some households in southern part of Nigeria reveals that they maintain the hygiene of the kitchens, cutting and cooking utensils, and also have their hands washed before touching foods in order to avoid food contamination. Rather than leaving food opened they ensured that all foods are covered or kept in the fridge or safe places. On another note is consciousness of some individuals about the safety of the food items they are purchasing based on NAFDAC sensitisation of the public on need to cross check manufacture and expiry dates on food and drink products. With this, many Nigerians become sure that they are consuming safe foods and drinks.

8. Conclusion

It is an established fact that food is of great value to man in that it sustains life, nourishes the body and supports healthy living. On this account a great deal of effort is made by man to ensure that food is readily available in sufficient quantity and quality for consumption. Farm cultivation though constitutes the primary source of food production, it becomes accessible to man either directly from cultivated farms or by purchase from the from the farm gates/markets. The value of food however goes beyond the satisfaction of hunger to nourishing the body for proper physiological growth and proper function of the body system. In this wise, nutritionally rich food becomes essential. That is, food to be consumed by individuals and/or households must be variety of food resources rich in carbohydrate, protein, fats, minerals and vitamins, coupled with sufficient water. While a larger proportion of the residents of the developed and affluent countries might have access to variety of foods, even in sufficient quantities, it is not the same case with most people in the developing countries as result of poor agricultural development and poor economic status of most individuals and households in the area. In Nigeria for instance, studies have revealed that most households in the country heavily relied on carbohydrate or energy-giving food due to poor state of the country's agriculture, low-income earning and poverty status of most households of its citizens. In addition to this is rationing of food by skipping one or two meals per day as a way to cope with food shortages or inability to afford adequate food for consumption. Thus, food consumption among most households in Nigeria is meant to satisfy hunger rather than nourish the body. On this account, the common dictum that 'a secured food person is out of poverty', is born out of the need to have something to eat, so far it is not a poison, rather than have nutrient-rich food to eat for nourishment of the body. In addition to this, the country generally lacked officially institutionalised food education programme for homes and families except subject of taught in formal school setting for education of school pupils and students at the basic education level and for training and development of food and nutrition professionals, most of whom end up working in hospitals and food industries as career officers or

personnel rather than food and nutrition educators for homes and families. In place of this, the age-long food consumption practice and traditional knowledge of food value and nutrients becomes the information-base for guiding food and nutrition education of the less educated individuals and households in Nigeria, while the Internet platform becomes the information source on food and nutrition for educated individuals and family care-giver that care to know about food and its nutritional value to man. On this, account, even though a large proportion of the Nigerian citizens or households lived below the poverty line and the country lacked sufficient food, appropriate development of food and education programme, coupled with integration of impactful teaching strategies, will go a long way to guide Nigerian on right choices of food selection and development of healthy food consumption behaviours. In this wise, conscious and critical look should be given to following food and nutrition education strategies for formal implementation in the country. The nutrition education strategies which are broadly classified as individual, group and environment approaches under the California Women, Infant and Children (WIC) Program include: the learner-centred approach, educator-centred counselling/advising, motivational interviewing, and self-learning/self-study strategies as the individual learning approach; child-centred approach, family-centred approach, interactive activities, role play, video presentation, facilitated group discussion, lectures, lectures with discussion, panel of experts and guest speaker strategies as the group approach; and the use of bulletin boards, educational material rounders/racks, music, posters, props and waiting room videos as the environment approach. Implementation of any of these food and nutrition education strategies requires proper planning

References

[1] Reference.com. Why do we need food to survive? [Internet]. IAC Publishing, LLC. 2017 [cited 2017 Apr 4]. Available from: https://www.reference.com/science/food-to-survive-16966e82d3d2cedc?qo=contentSimilarQuestions

[2] Pardey P, Beddow, J. Agricultural innovation: the United States in a changing global reality. 2013. Chicago, USA, The Chicago Council on Global Affairs.

[3] FAO. World agriculture: Summary report. Food and Agriculture Organization of the United Nations, Rome. 2002.

[4] Food Security Portal. Nigeria: Overview [Internet]. Food Security Portal facilitated by International Food Policy Research Institute – IFPRI [cited 2017 Jun 21]. Available from: http://www.foodsecurityportal.org/nigeria

[5] FAO. Nigeria at a glance. http://www.fao.org/nigeria/fao-in-nigeria/nigeria-at-a-glance/en/

[6] Edo State Agricultural Development Programme . Pre-participating rural appraisal for need assessment of form of special food security 2002.

[7] Punch. Nigeria spends $11bn yearly on food importation [Internet]. Punch News Paper, 2016, July 14. [cited 2017 Mar 23]. Available from: http://punchng.com/nigeria-spends-11bn-yearly-food-importation/

[8] Ojo EO, Adebayo PF. (2012). Food security in Nigeria: An overview. *European Journal of Sustainable Development*, 2012, 1(2), 2239-5938

[9] Food Security Portal:

[10] Omorogiuwa O, Zikovic J, Ademoh F. The role of agriculture in the economic development of Nigeria. *European Scientific Journal.* 2014; 10(4): 133-147.

[11] FAO. Roots, tubers, plantains, and bananas in human nutrition. Food and Agriculture Organization of the United Nations, Rome, 1990.

[12] Salvador EM, Steenkamp V, McCrindle, CME. Production, consumption and nutritional value of cassava (Manihot esculenta, Crabtz) in Mozambique: An overview. *Journal of Agricultural Biotechnology and Sustainable Development.* 2014; 6(3), 29 – 38.

[13] Washaya S, Mupangwa JF, Muranda E. Nutritional value and utilisation of yams (Dioscorea steriscus) by residents of Bindura Town High density Suburbs, Zimbabwe [Internet]. *Advances in Agriculture,* 2016. 7pp. [cited 2017 Mar 23]. Available from: http://doi.org/10.1155/2016/5934738

[14] Bankole YO, Tanimola AO, Odunukan RO, Samuel DO. Functional and Nutritional Characteristics of Cassava Flour (Lafun) Fortified with Soybeans. *Journal of Educational and Social Research.* 2013; 3(8): 163 – 170

[15] McKevith B. Nutritional aspects of cereals [Internet]. British Nutrition Foundation Nutrition Bulletin. 2004; 29, 111–142. [cited 2017 Mar 23]. Available from: http://doi.org/10.1155/2016/5934738

[16] Goldberg G. Plants: *Diet and Health.* The Report of the British Nutrition Foundation Task Force. Blackwell, Oxford, 2003.

[17] Kulp K, Ponte JG. Handbook of Cereal Science and Technology, 2nd edn. Marcel Dekker, New York. 2000

[18] Arnarson D. Rice 101: Nutrition facts and health effects . [Internet]. 2017. [cited 2017 Mar 23]. Available from: https://authoritynutrition.com/foods/rice/

[19] Singh KP, Mishra A, Mishra HN. Fuzzy analysis of sensory attributes of bread prepared from millet-based composite flours. LWT-Food Sci Technol. 2012; 48: 276 – 282.

[20] Saleh A. SM, Zhang Q, Chen, J, Chen Q. Millet grains: Nutritional quality, processing, and potential health benefits. Comprehensive Review in Food Science and Food Safety. 2013; 12(3): 281 – 295.

[21] Venter CS, Eyssen E. More Legumes for Overall Better Health. Dry bean Producers Organisation, Pretoria. *SAJCN (Supplement)* 2001; 14(3):S32-S38.

[22] Boschin G, Arnoldi A. Legumes are valuable sources of tocopherols. *Food Chemistry.* 2011; 127:1199-1203.

[23] NAPC/IRR. Integrated community food production: A compendium of climate-resilient agriculture options. 2016. National Anti-Poverty Commission (NAPC) and the International Institute of Rural Reconstruction (IIRR): Philippine

[24] Gopala. C, Rama Sastri BV, Balasubramanian SC. Nutritive value of Indian foods [Internet]. National Institute of Nutrition ICMR, Hyderabad. 2004 [cited 2017 Mar 23]. Available from: .http://webcache.googleusercontent.com/

search?q=cache:9wUSuIjPlikJ:agritech.tnau.ac.in/nutrition/pdf/nutritive%2520value_milk.pdf+&cd=19&hl=en&ct=clnk&gl=ng
[25] AgriInfo [Internet]. 2015. [cited 2017 Mar 23]. Available from: http://www.agriinfo.in/?page=topic&superid=2&topicid=1011
[26] Nnakwe N, Onyemaobi G. Prevalence of food insecurity and inadequate dietary pattern among households with and without children in Imo State Nigeria. *International Journal of Sociology and Anthropology.* 2013; 5(9); 402-408
[27] Olarinde LO, Kuponiyi FA. Rural livelihood and food consumption patterns among households in Oyo State, Nigeria: Implications for food security and poverty eradication in a deregulated economy. J. Soc. Sci. 2005. 11(2): 127-132
[28] Nagataab J, Magerengec R, Youngd S, Ogutac S, Weisere S, Cohenf C. Social determinants, lived experiences, and consequences of household food insecurity among persons living with HIV/AIDS on the shore of Lake Victoria, Kenya. *AIDS Care-Psy and Socio-Med Asp of AIDS/HIV*. 2012: 24 (6): 728-36.
[29] Ogunkunle MO, Oludele AS. Food intake and meal pattern of adolescents in school in Ila Orangun, south-west Nigeria. S Afr J Clin Nutr. 2013; 26(4):188-193
[30] Igbedoh SO. Undernutrition in Nigeria: Dimension, Causes and Remedies for Alleviation in a changing socio-economic environment [cited 2017 May 21]. N*utrition and Health,* 1993; 9(1), 1-14. [cited 2017 May 21]. Available from: http://journals.sagepub.com/doi/pdf/10.1177/026010609300900101
[31] Müller O, Krawinkel M. Malnutrition and health in developing countries. *CMAJ.* 2005; 173(3): 279-286
[32] Moore, S., Collinson, A., N'gom, P., Aspinall, R., & Prentice, A. Early immunological development and mortality from infectious disease in later life. Proceedings of the Nutrition Society, 2006; 65, 311-318.
[33] McGregor, S., Cheung, Y., Cueto, S., Glewwe, P., Richter, L., & Strupp, L. Developmental potential in the first 5 years for children in developing countries. *Lancet,* 2007; 369, 60-70.
[34] Glewwe, P., Jacoby, H., & King, E. Early childhood nutrition and academic achievement: A longitudinal analysis. *Journal of Public Economics,* 2001; 81, 345-368.
[35] MHETEC/FAO. Food & Nutrition: A Handbook for Namibian Volunteer Leaders. Ministry of Higher Education, Training and Employment Creation, Namibia and Food and Agriculture, Organisation, Rome; 2004.
[36] Brabin BJ, Coulter JBS. Nutrition-associated disease. In: Cook GC, Zumla AI, editors. Manson's tropical diseases. London: Saunders; 2003. pp. 561-80.
[37] Rice AL, Sacco L, Hyder A, Black RE. Malnutrition as an underlying cause of childhood deaths associated with infectious diseases in developing countries. Bull World Health Organ 2000;78:1207-21.
[38] Müller O, Garenne M, Reitmaier P, van Zweeden A, Kouyate B, Becher H. Effect of zinc supplementation on growth in West African children: A randomized double-blind placebo-controlled trial in rural Burkina Faso. *Int J Epidemiol.* 2003;32:1098-102.
[39] Mwaniki A. Iron deficiency in Bangladesh. In P. Pinstrup-Andersen and F. Cheng (Eds). Food policy for developing countries: The role of government in the global food system. Case study #3-3 of the program. 2007. pp 1 – 9. Cornell University
[40] Fleming AF, de Silva PS. Haematological diseases in the tropics. In: Cook GC, Zumla AI, editors. Manson's tropical diseases. London: Saunders; 2003. p 169-244.
[41] Ghana VAST Study Team. Vitamin A supplementation in northern Ghana: effects on clinic attendances, hospital admissions, and child mortality [published erratum appears in Lancet 1993;342(8865):250]. Lancet 1993;342:7-12.
[42] Müller O, Jahn A, von Braun J. Micronutrient supplementation for malaria control — hype or hope? [editorial]. *Trop Med Int Health* 2002; 7:1-3.
[43] Levin HM, Pollitt E, Galloway R, McGuire J. Micronutrient deficiency disorders. In: Jamison DT, Mosley WH, Measham AR, Bobadilla JL, editors. Disease control priorities in developing countries. 2nd ed. Oxford (UK): Oxford University Press; 1993. pp. 4 21-451.
[44] Sazawal S, Black RE, Bhan MK, Bhandari N, Sinha A, Jalla S. Zinc supplementation in young children with acute diarrhea in India. *N Engl J Med* 1995; 333:839-44.
[45] Agee MD. Reducing child malnutrition in Nigeria: Combined effects of income growth and provision of information about mothers' access to health care services. *Social Science & Medicine.* 2010; 71, 1973-1980.
[46] UNICEF-Nigeria. Nutrition: The situation [Internet]. The United Nation Children Education Fund. 2017. [cited 2017 May 22]. Available from: https://www.unicef.org/nigeria/nutrition_147.html
[47] McNulty, J. Challenges and issues in nutrition education [Internet]. Rome: Nutrition Education and Consumer Awareness Group, Food and Agriculture Organization of the United Nations 2013, [cited 2017 April 11]. Available from: www.fao.org/ag/humannutrition/nutritioneducation/en/
[48] Pelletier DL, Frongillo Jr, EA, Schroeder DG, Habicht JP. The effects of malnutrition on child mortality in developing countries. Bulletin of the World Health Organization, 1995, 73 (4): 443-448
[49] Gil A. 2010. Tratado de Nutrición: Tomo III - Nutrición Humana en el Estado de Salud (2nd edition, 3rd Volume). Madrid: Editorial Médica Panamericana, S.A.
[50] Contento IR. Nutrition Education: Linking Research, Theory, and Practice 2nd edn. Sudbury, MA: Jones and Bartlett; 2011
[51] ADA. International Dietetics and Nutritional Terminology - Reference Manual. Standard Language for the Nutrition Care Process 3rd edn. Chicago, IL: American Dietetic Association. 2011.
[52] Eat Well. Review of policy actions, data available for their analysis and existing evaluations throughout Europe [Internet]. Eat Well; 2011 [cited 2017 May 14]. Available from: Available from: eatwellproject.eu/en/Eatwell-research/Project-Reports/
[53] Nutrition Education (n.d.) Wisconsin Wellness: Putting Policy into Practice [Internet]. Wisconsin University. [cited 2017, Mar 23].Available from: http://www.epi.umn.edu/let/pubs/img/adol_ch5.pdf
[54] Kalmus, S. Nutrients in Vegetables After Blanching [Internet]. Livestrong/com, 2015. [cited 2017 Jun 23]. Available from http://www.livestrong.com/article/528167-nutrients-in-vegetables-after-blanching/
[55] Food and Nutrition Service. Nutrition education and promotion: The role of FNS in helping low-income families make healthier eating and lifestyle choices – A Report to Congress [Internet]. Office of Research and Analysis: United State Department of Agriculture. 2010. [cited 2017 May 14].

Formal and Non-Formal Methods in Food Chemistry and Engineering Education

H. Kautola[1], **K. Hölttä**[2] and **S. Mahlamäki-Kultanen**[3]

[1] Biotechnology and Food Engineering, School of Bioeconomy, Häme University of Applied Sciences, Visamäentie 35A, FI-13101 Hämeenlinna, Finland
[2] Mentoring Programme and Alumni Relations, Aalto University, 00076 Aalto, Finland
[3] School of Professional Teacher Education, Häme University of Applied Sciences, Visamäentie 35A, FI-13101 Hämeenlinna, Finland

Mentoring in learning has been adopted lately as one of the near-work education method. In the virtual mentoring, either individual or group mentoring, the learning and development of the actors, mentees, may increase a lot with the help of other participants and their experiences. [1] The mentor-actor relationship can supervise the actor to a path that fulfils the wishes and future for a remarkable goal of career [2]. The pedagogical strategy framework was used to find the critical points of the structure in the learning process and to find the best digital methods to use in the chosen *team learning system*.[3,4] The knowledge of food sciences of the mentors teaches the profession of, for instance, the food chemistry and engineering for the actors. The peer group mentoring (PGM) is a tool to the career development [5]. The networking and mentoring improves training, develops competencies and new learning environments, as well as the collaborative relationships between novice and experienced actors.

Keywords: professional education, mentoring, PGM, food

1. Introduction

As work is changing, also the education methods and learning spaces as well as facilities must provide new possibilities for the modern education systems. The staff in food industry is widely spread around in the countryside, also in the cities. This causes needs for flexible learning in several locations at the same time. To reach proper education in food sciences means that the education methods must meet the needs for several varieties of skills. Digital learning methods together with novel pedagogical methods are expected to bring for the younger Y-, Z-generation, and the present generation, easier ways to reach the needed education.

Web-based learning methods have developed fast the last decades. The social media tools and hardware development has challenged the pedagogical side to find the best learning ways to enhance good competences in food sciences. Project and team learning, with the help of mentoring, is an easy application for the use of new tools, mobile centers and new digital hardware. The diginatives have special expectations for learning methods independent on place.

It is a challenge to ensure teaching and mentoring quality in new types of learning environments keeping up the standard of professionals also in the 21st century. New food factories and new learning environments have created a huge variety of job opportunities for newly qualified professionals. eMentoring is a vehicle to sharpen ones' professional skills. [6] There is a difference between the idea of benefits in relationships between mentors and mentees. Mentees' benefits are clear, but the mentees do not find the benefits for the mentors. [7] Mentoring as a tool in educating in higher education institution courses has not been published a lot, yet.

2. Methods

In methodology we need to separate two definitions: *coaching and mentoring*. Here, coaching is understood to include active guidance to lead to one's career path, where mentoring is more following the actor, mentee, and giving advice when needed in a form that the mentee needs to find the solution him/herself. The mentor gives examples from real life that has happened and what has been the consequence of such case and helps in, for instance, making analyses by making questions. The tools used in mentoring are pair meetings, seminars, visits to different organisations, peer mentee group evenings, round table discussions, and thematic workshops.[2]

The pedagogical strategy framework was used to find the critical points of the structure in the learning process and to find the best digital methods to use in the chosen *team learning system*. Options of using digital tools like mobile center was the mentees' own choice. Only the learning platform was fixed by the mentor/teacher and institute. Feedback was used for developing the course in the future. The motivation of students was measured by interviews. Those not willing to learn new tools were also activated by other team members. [3,4] The method was used for Food Industrial Plant Design course with 6 groups.

Peer group mentoring (PGM) has also been a tool for teacher career development.[5] In the action research the newcomers and experienced educators were mixed in the same group to give the possibility to develop by peer group mentoring. The new teacher will be boosted into creating new infrastructure for modern learning purposes. The method

was used in food chemistry course carried out with 10 teachers of 2 experienced and 6 newcomers. Both type of teachers participated in equal conditions during the sessions.

3. Results

Mentoring in learning has been adopted lately as one of *the near-work education method*. In the virtual mentoring, either individual or group mentoring, the learning and development of the actors may increase a lot with the help of other participants and their experiences. [1] The mentor-actor relationship can supervise the actor to a path that fulfils the wishes and future for a remarkable goal of career [2]. The knowledge of food sciences of the mentor teaches the profession of, for instance, food chemistry and engineering for the actor, mentee.

It is important to remember that the mentor does not necessary need to know everything, and this is why uncertainty is a common feeling. The mentee needs to be active with questions, bringing up both positive and negative things during the process. It is important to make a good plan for the meetings like in **Table 1**, to get an efficient mentoring session, as time is a limiting factor. The goals in learning must be defined in the beginning of the mentoring programme.

In the peer group mentoring system the teacher students developed some question at the beginning. They wanted to find out first the theoretical principles, the relations between the experiment and course programme, and the goal of the experiment. An important role was also with the human and cost resources and logistic and time needed. Most important was the safety conditions to consider when food chemistry was the subject. All these matters were widely discussed during the practical experiment in the mixed peer group mentoring (PGM).

In the team learning system, a web-based course in food process design was produced. The food industry was interviewed to find the needs in present knowledge and in the near future (soft system methodology), suitable to be solved during the study course of food industry plant design. The pedagogical strategy framework was used to define the critical points in the designing process and to find the best digital methods to use in the system, shown in **Table 1**. The students from two universities were given options of using tools like dropbox, facebook, and digital tools like mobile center of their own choice. The learning platform was fixed by the teacher and institute. Feedback received from 6 study groups of 8 students in 3 years was: first year depressing (too much work), second year active, and third year constructive and willing to continue the method and development. The groups were each year different and the information from the previous years' groups, also the influence of the attitude change on new study methods probably revised some of the opinion. The motivation of students increased and those not willing to learn new tools were also activated by other team members.

Table 1 The pedagogic plan for the Industrial Food Process Design e-course [3, 8].

The aim of the course	Contents	Learning, the student performance	Steering and feedback, the teachers performance	References of knowledge	The tools for eworking
Aims as competences	Description of the contents	How does the student learn this? LbD.	How and when the work is being supervised? (pedagogic, social, administartive and technical support)? Supervision throught moodle or webex. Meetings e-conference or face-to-face. Seminars throught webex (camera and mic).	Produced before or during the process? In advance will be given the frame of the task in parts according to the project schedule. Also the students study information and collect material for the task. Finally there will be a final report collected from information in the intermediate reports.	What net tools to use to support the work? First finding out what tools are available and suitable to use? Moodle and cad and other programmes, ebeam, webex (acp), skype, confluence-wiki, mobile center, free social media tools, webropol, etc.
Aim 1: Knows how to design a preparatory plan.	Content 1: Basic knowledge for designing the preparatory plan, working hour plan for follow up in schedule control.	How does the student work? Team working and in smaller groups. Networking.	Who supervises and gives feedback? The teachers give oral feedback during the meetings.	Ready printed material or teacher written material? Both.	The learning platform and its tools? Moodle, real-time net tools for communication, blogs, wikis, intranets; sms, e-mail, phones/mobiles, skype, mobile center
Aim 2: Knows how to read and draw process diagrammes	Content 2: Drawing process diagrams, making lists and	What kind of learning assignments? Larger project work divided into smaller parts.	Planning the teachers' and tutors' time schedule and amount of work.	How to use different media elements: text, sound/voice, picture, grafics, animation,	

and understands their differences.	specifications and writing intructions.			video, database? Plenty of database, pictures in diagramme production.	
Aim 3: How a larger project is performed in a team.	Content 3: Dividing work, taking in account expertise of others, reporting.	The students and the groups performance? Team work, team leader and secretary in each turn, learning meeting technology.	The teacher working hours according to face-to-face meeting sessions, theory lessons and practises.	How to use study sources? The project will be done in parts and will be presented as a seminar and a final report.	
		The plan of students' time schedule and amount of working hours? This plan will be created at the beginning of the course: Estimate and implementation table.			

4. Conclusion

Peer Group Mentoring (PGM) can be used to develop the careers of food science experts. PGM shows how networking and mentoring can improve the training of experts in food sciences. They can develop competencies, build new learning environments and study the quality for analysis, logistics, and skills needed for risk assessment. The pilot case was studied among food chemistry technicians. The participants recognized the importance of collaborative work for the actor.

In food industries where changes in the organisations are needed, it is easier to fulfil the tasks with educated employees who have learned the similar view of the consequences.

In team learning system the motivation of students was higher than in individual learning methods and those not willing to learn new digitools were also activated by other peer team members. The competence learning was deeper and more efficient and economical. The earlier used study systems and methods, as well as the previous study groups opinions gave some influence on the feedback.

References

[1] Leppisaari I, Mahlamäki-Kultanen S, Vainio L. Virtuaalinen ryhmämentorointi ammattikorkeakoulu-opettajan kehittymisen tukena. (Virtual group mentoring supporting the development of HEI-teacher). Aikuiskasvatus (Adult Education), 4/2008, 278-287. (in Finnish)

[2] Aalto yliopiston mentorointiopas (Mentoring Guide of Aalto University). Mentori, Mentorointi, Mentoroitava (Ed. K Hölttä) Aalto yliopisto Kumppanuuspalvelut. 2012, Helsinki.

[3] Kautola HK. Yritysyhteistyön integrointi prosessisuunnittelun opetukseen Hämeen ammattikorkeakoulussa: Jätevedenpuhdistamo (Industrial collaboration integration into process design teaching at Häme University of Applied Sciences: Wastewater treatment plant.). In: Suomi nousuun insinööriosaamisella (eds. Lankinen, P., Rask, M. and Savander-Ranne, C.) Metropolia Digipaino, Espoo 2015, pp.75-79. (in Finnish) http://www.metropolia.fi/fileadmin/user_upload/M.fi-2015/Palvelut/Julkaisut/MET_inssiforum_04092015.pdf

[4] Kautola HK, Pirttijärvi T, Kullaslahti J, Majuri M. Pedagogical Aspects in Using SOME at Education for Food Industry. Abstracts *16th World Conference of Food Science and Technology*, Aug. 5-9, 2012, Foz du Iguassu, Brazil. Number 262.

[5] Page J, Mahlamäki-Kultanen S, Luis A, Bento R, Costa M and Íris A. Peer Group Mentoring for Teacher Development at Brazilian Vocational Education Network: A Pilot Project under a Brazil – Finland Partnership. Ammattikasvatuksen aikakauskirja - Journal of Professional and Vocational Education, 2017 (submitted).

[6] IFT- eMentor. http://www.ift.org/careercenter/ementor.aspx, 24.4.2017

[7] Campbell DE, Campbell TA. The Mentoring Relationship:Differing Perceptions of Benefits, College Student Journal. 34 (4) (2000)516- 524. http://search.ebscohost.com/login.aspx?direct=true&db=afh&AN=4044540site=ehost-live

[8] Kautola, HK, Pirttijärvi, T, Kullaslahti, J, Laitinen, E. Pedagogy in Digital Learning in Food Eduction. *5th African Higher Education Week and RUFORUM Biennial Conference*, Cape Town Oct. 17-21, 2016. *RUFORUM Working Document Series* (ISSN 1607-9345) No. 14 (1) 2016: 271-276. *http://repository.ruforum.org/ag_documents-catalog?keys=Pedagogy+in+Digital+Learning+in+Food+Education*

Fructan rich diet to improve gut microbiota in disease and health

Merve Erginer Haskoylu, **Songul Yasar Yildiz** and **Ebru Toksoy Oner**

IBSB-Industrial Biotechnology and Systems Biology Research Group, Department of Bioengineering, Marmara University, Istanbul, Turkey

Keywords: dietary fiber; microbiota; fructan; FOS; levan

1. Microbiota

Microbiota is a community of microorganisms that populate a certain environment and specially the population of microorganisms inhabiting in or on the human body. Human body forms a platform on which diverse microbial ecosystems are established. After the birth of a mammal, a life-long process of colonization by foreign microorganisms that inhabit most environmentally exposed surfaces such as the skin, mouth, gut and vagina is initiated [1, 2]. The human microbiota involves more than 10^{14} symbiotic microbial cells [3] and 10^{15} viruses [4] harbored by each person, predominantly bacteria in the gut; the human microbiome consists of the genes these cells harbor [5, 6]. In short, the microbial population that inhabits in and on the human body establish our microbiota, and the genes they encode are defined as our microbiome. This complex community involves bacteria, eukaryotes, viruses, and at least one archaeon that interact with one another and with the host, greatly impacting human health and physiology. Cultivation of these microorganisms are difficult and only small number of them can be cultured. However, culture-independent high-throughput sequencing provides a great information about these microorganisms [3, 7]. Many samples can be characterized and compared rapidly by the highly multiplexed studies [8, 9]. These studies enable the detection of spatial, temporal, and disease-associated patterns in human microbiota. In humans, the microbiota plays a significant role in health and disease and it can be occasionally referred to as our ‘‘forgotten organ’’ [10]. The microbiota takes a role in energy harvest and storage, besides its fundamental role in a variety of metabolic functions such as fermenting and absorbing undigested carbohydrates [11]. Most significantly, the gut microbiota interacts with the immune system and provides signals for the maturation of immune cells and the normal development of immune functions [12].

2. Gut Microbiota

The microbiota colonization occurs on every surface of the human body that is open to the external environment. Microorganisms live in our skin and in the genitourinary, gastrointestinal, and respiratory tracts [13-15]. The gastrointestinal tract (GIT) is the most colonized organ; only the colon contains more than 70% of all the microorganisms in the human body [1, 3]. The human gut has 200 m^2 surface [16] that represents a significant surface for microbial colonization. Moreover, the GIT is a favored surface for colonization since it provides a variety of nutrients for microbes. Strict anaerobes are dominant in gut microbiota with respect to the facultative anaerobes and aerobes [17-19]. The gut microbiota of healthy individuals is composed of six bacterial phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* [20]. However, only the *Bacteroidetes* and the *Firmicutes* dominate in the human gut microbiota [21]. Variety of bacterial species in the human gut reaches as much as 35,000 species [22].

The GIT has non-homogenous microbiota. The number of bacterial cells in stomach, duodenum, jejunum, ileum and column are approximately 101, 103, 104, 107 and 1011 bacteria per gram, respectively [10]. Furthermore, the composition of microbial community differs in different parts. Biopsy samples from colon and small intestine of healthy people were compared and enrichment of different bacterial communities were observed at these two sites [22]. Bacilli class of the *Firmicutes* and *Actinobacteria* were dominant in small intestine, however; *Bacteroidetes* and the *Lachnospiraceae* family of the *Firmicutes* were enriched from colonic samples. Furthermore, there is also difference in microbiota of intestinal lumen between attached and embedded in the mucus layer which separates intestinal epithelium from the lumen. For example, *Bacteroides*, *Bifidobacterium*, *Streptococcus*, members of *Enterobacteriacea*, *Enterococcus*, *Clostridium*, *Lactobacillus*, and *Ruminococcus* were all detected in feces, on the other hand, only *Clostridium*, *Lactobacillus*, and *Enterococcus* were found in the mucus layer and epithelial crypts of the small intestine [23].

Bacteria in the GIT can be divided into two main groups: useful bacteria (good bacteria) and harmful bacteria (bad bacteria) which must be kept in the intestine (Fig. 1). Numerous factors can affect gut flora and it has been linked to various diseases including obesity, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), non-alcoholic steatohepatitis (NASH), colon cancer, liver cancer, type II diabetes mellitus, allergic diseases and cardiovascular diseases [24, 25]

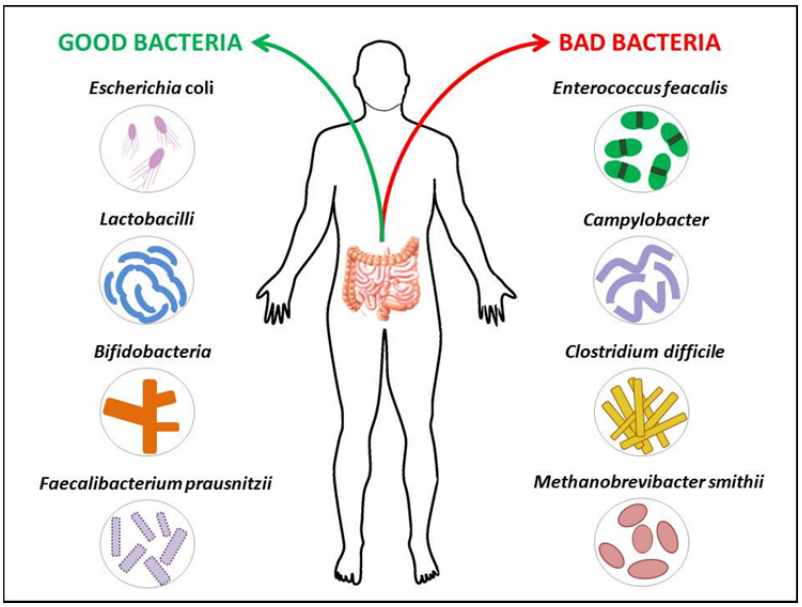


Fig. 1 Bacterial flora of gut.

Formation of microbiota in the human gut starts with the birth. During the canal of birth, infants are exposed to varying microbial groups [26] in the vaginal microbiota of their mothers, similarity between microbiota of infants intestine and that of mother vagina is an evidence for this colonization in the infant during the birth [27]. Moreover, different microbial populations were observed from the infants delivered through cesarean section [28]. Microbiota of intestine during the first year of baby is very simple, but it can change from individual to individual and from time to time. However after the first year, gut microbiota of baby becomes stable and similar to that of a young person [27, 29]. Microbial community of individual GIT was affected by many other factors such as host physiology, genetics, and environmental factors [30, 31]. Increasingly, diet is recognized as a key environmental factor that mediates the composition and metabolic function of the gastrointestinal microbiota [32]. Indeed, consumption of specific dietary ingredients, such as fiber and prebiotics, is an avenue by which the microbiota can be modulated.

3. Dietary fibers and prebiotics

Dietary fibers are defined as [33]: carbohydrate polymers with ten or more monomeric units, which are neither digested nor absorbed in the human small intestine and belong to the following categories: (i) edible carbohydrate polymers naturally occurring in foods as consumed, (ii) edible carbohydrate polymers which have been obtained from food raw materials by physical, enzymatic, or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence, and (iii) edible synthetic carbohydrate polymers which have a beneficial physiological effect demonstrated by generally accepted scientific evidence. Prebiotics are also classified as dietary fibers. Prebiotics are recently defined as according to International Scientific Association of Probiotics and Prebiotics (ISAPP) [34]: "selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health."

Target genera of prebiotics are dominantly *Lactobacilli* and *Bifidobacteria*. When two genera were compared, changes were observed more often in *Bifidobacteria* since more *Bifidobacteria* usually reside in the human colon than *Lactobacilli*, and they exhibit a preference for oligosaccharides [35]. To classify an ingredient as a prebiotic, it should resist gastric acidity, be hydrolyzed by mammalian enzymes, absorbed in the upper GIT, be fermented by the GIT flora, selectively stimulate the growth/activity of GIT bacteria potentially associated with health and well-being. The known health benefit for prebiotics intake is rather limited than for dietary fibers. However, it has been put forward that prebiotic intake may ; reduce inflammation and inflammatory bowel disease symptoms, duration and frequency of antibiotic-associated diarrhea, lower the risk of cardiovascular diseases, protect against colon cancer, enhance absorption and bioavailability of calcium, magnesium, iron minerals and prevent obesity while supporting weight lost and satiety [35].

New technologies in molecular and computational studies show the effect of diet on composition and function of GIT microbiota. For instance, metagenomics provide the information for gastrointestinal microbiome which is 150 fold more than genome of the human [11]. Most fibers and prebiotics cannot be digested by the human enzymes, less than 20 glycosidases involved in digestion of dietary polysaccharides have been recognized in the human genome [36]. From this perspective, metabolization of dietary polysaccharides by the gastrointestinal bacteria is an example of the symbiotic relationship between the host and the microbiota. Furthermore, this relationship provides an opportunity for dietary modulation of the microbiota since microbial growth and metabolism depend on substrate availability, i.e., the type of dietary fiber or prebiotic consumed by the host.

4. Fructans as prebiotics

Fructans are non-structural carbohydrates that occur in bacteria, algae, flowering plants and mosses [37]. Fructans are fructose-based oligo- and polysaccharides containing maximal one glucose unit [38]. They are the most common and known group among prebiotics and can be divided into five main groups based on their chemical structure. (i) inulin

with β-(1-2)linked fructose residues, (ii) neo-inulin with two β-(1-2)-linked fructose chains attached to the sucrose starter unit, (iii) β-(2-6) linked levan, (iv) neo-levan with internal glucose residue and (v) graminans (mixed fructans) consist of β-(2-6)-linked fructose residues with β-(1-2) branches [37, 39].

Fructans provide improvement of blood parameters, resistance against intestinal and extra-intestinal pathogens, modulation of immune system and reduced allergies [40]. The intestinal benefits of fructans as well as their symbiotic association with probiotic bacteria, encompass prevention and treatment of infectious diseases, including viral or bacterial diarrhea, and chronic inflammatory diseases such as ulcerative colitis [41]. It is known that their prebiotic activity changes with structural features like; composition of monomers, type of glycosidic linkage, degree of polymerization (DP) and degree of branching [42].

Prebiotic fructans cannot be digested in stomach and small intestine on the other hand, might be partially hydrolyzed due to acidic environment [37]. When they reach colon, glycoside hydrolase (GH32) secreting bacteria degrade them and they serve as bifidogenic factor on beneficial probiotic bacteria and can be used as carbon and energy source [43] At the end of this metabolic activity, short chain fatty acids (SCFAs) like butyrate, propionate, acetate, lactate and hydrogen, methane, and carbon dioxide are produced. SCFAs are used as carbon and energy source for bifidogenic bacteria and play important role in the enhancement of host health through enhancing immune system, facilitating mineral uptake, neuronal and hormonal feedbacks [40, 43]. Butyrate is known to increase growth of *Lactobacillus* and *Bifidobacteria* [44]. Moreover, butyrate plays important role on epithelial cell proliferation and differentiation [37]. Acetate join circulation and goes to liver and it is used as energy source while propionate is absorbed and metabolized aerobically and play key role in many mechanisms for instance appetite regulation. [45, 46]. It is reported that FOS play a key role in production of butyrate and propionate in high amounts [47]. Long term effects of FOS consumption is studied by Le Blay et al. [48], and rats fed with diet containing FOS (9-10 g/day) during 27 weeks and they reported transitory increase in lactate and constant increase in butyrate.

4.1 Inulin

Inulin is a β-(1-2) linked fructose polymer generally with terminal glucose and its DP varies from 2 to 60 units [49, 50]. Inulin can be synthesized enzymatically from sucrose or extracted mainly from plants. Inulin type fructans are generally obtained from chicory (*Cichorium intybus L*) or artichoke (*Helianthus tuberosus*) but recently Agave cactus and oats are also considered as rich soruce of inulin fructans [51, 52]. It is reported that inulin based dietary fiber should contain at least 3-6 g /100 g inulin while 3-8 g of inulin per portion should be consumed for bifidogenic activity [47].

4.2 Fructooliosaccharides

Fructose oligomers with low DP are called as fructooligosaccharides (FOS). FOSs originate from sucrose molecules and main monomeric units are fructose [47, 53]. Inulin type FOS and levan type FOS are two different types of FOS [54]. Partially hydrolysis of FOSs generate oligofructoses like glucopyranosyl-(fructofuranosyl)n-1fructose (GFn) and fructopyranosyl-(fructofuranosyl)n-1 fructose (Fn) with different DP degrees (1-4) [55]. 1-kestose (GF2), nystose (GF3), fructofuranosyl nystose (GF4) are also called FOS because of fructose monomers present in their composition [56]. Oligofructans are synthesized by hydrolysis of long fructan chains or sucrose based enzymatic synthesis. Inulin type oligofructans can be obtained from hydrolysis of inulin by endo-inulinase (EC 3.2.1.7) [57] or synthesized from sucrose by *Aspergillus aculeatus* derived fructosyltransferase (EC 2.4.1.9) [58] , *Aspergillus niger* derived β-fructofuranosidase (EC 3.2.1.26) enzymes (58)**.** FOSs can be found commonly in chicory, Jerusalem artichoke, asparagus and onion family members [59]. Daily consumption amount for bifidogenic activity is reported as 2-10 grams [60]. FOS can be used for prevention of intestinal infections and intestinal infections; inhibition of pathogens, ordering intestinal flora; regulation of intestinal immune system; enhancement of immune response; stimulation of probiotic growth of *Lactobacilli* and *Bifidobacteria* species; optimization of colonic function and metabolism; production of short chain fatty acids; increase of mineral absorption; reduction of food intake and obesity management and control of diabetes type 2 and prevention of cancer [41, 54, 61-72].

4.3 Levan

Levan is β-(2-6) linked fructose polymer commonly found in nature. Water soluble, nontoxic and strongly adhesive polymer have many application fields such as pharmacy, food, chemistry, medical and cosmeceutical industries [73]. It is produced extracellularly from sucrose based substrates by various microorganisms including first extremophilic levan producer *Halomonas smyrnensis AAD6T* [74]. Several studies with levan as antioxidant, anticancer, anticoagulant, biocompatible microcarrier for peptide and protein drugs, adhesive multilayer films, bioactive surfaces, anti-irritant, prebiotic activities, antidiabetic and lipid metabolism regulator are reported by various researchers [74, 75]. Levan is a non-toxic soluble dietary fiber and hydrolysates of levan improve function of gut. Linkage type, length of polysaccharide and branching are significant physiological parameters that affect fermentation of levan in GIT. *In vitro* studies indicate enrichment of *Bifidobacterium adolescentis*, *B. pseudocatenulatum*, *B. breve*, *B. longum*, *Lactobacillus plantarum* and *Pedicoccus pentosaceus* with levan oligosaccharides [66, 76].

5. Fructans rich diet in health

5.1 Studies on prebiotic activity of fructans

Prebiotics are reported as more effective and simpler modulators on gut microbiota compared to probiotics because they stimulate the life cycle of the resident gut microbiota [37]. Fructans show their prebiotic activities through toll like receptors (TLR) and their fermentation products show their activity through AMPK and/or nuclear factor kappa B (NF-κB) signaling pathways [40]. Effect of inulin type fructan rich Jerusalem artichoke tuber on 72 wistar rats for 12 weeks is investigated by Samal et al. [77]. Increase of beneficial bacteria in colon and rectal digesta (*Lactobacillus* spp. and *Bifidobacterium* spp.), acetic acid, propionic aicd and total SCFA concentration and improved fiber digestibility observed. Chung et al [78] studied prebiotic effect of inulin and apple pectin *in- vitro*. Increased *Bacteroides*, *Eubacterium eligens* is observed for inulin. Prebiotic effect of Agave inulin (BIOAGAVE) (0, 5, 7.5 g/day) on 29 healthy humans for 3 weeks is observed by Holscher et al. [79]. Concentration dependent increase in *Bifidobacterium adolescentis*, *B. breve, B. longum, and B. pseudolongum* is observed while *Desulfovibrio* and *Clostridium sp*. decreased. Majid et al. [80] studied prebiotic activity of oligofructose and inulin containing fiber-prebiotic enriched solutions (7 g/day) (Nutrison Protein Plus Multifibre, Nutricia UK) on 22 diarrhea enteral nutrition fed patients for 7 days. No bifidogenic effect is observed but *Faecalibacterium prautsnizii* and *Bacteroides*/*Prevotella* decreased in fecal samples. *Bacillus subtilis* natto CCT 7712 FOS is investigated by Silva et al. [81] for its prebiotic activity. FOS was recorded as a good energy source. Highest growth on FOS was observed for *Lactobacillus plantarum* ATCC 14917 followed by *Lactobacillus casei* (LC-1). Study concluded that FOS and probiotics (*Lactobacillus casei* and *Lactobacillus plantarum* ATCC 14917) could be used as symbiotic in foods. Martine-Gutierrez et al. [82] investigated prebiotic effect of *Agave salmina* fructooligosachharides and inulin (ORAFTI®). Growth of *Lactobacillus acidophilus* is observed at the highest level in presence of *Agave salmina* FOS with lowest pH and highest lactate production. Porras-Dominguez [83] investigated prebiotic activity of levan-type oligofructans hydrolyzed by endo-levanase (EC 3.2.1.65) and high molecular weight levan and reported that levan-type oligofructans improve growth of Bifidobacteria more in comparison to levan. Commercially available branched levan (from *Erwinia herbicola* and *Lactobacillus sanfranciscensis* LTH 2590) were investigated for their prebiotic activities and *L. sanfranciscensis* levan showed bifidogenic activity [84, 85]. Prebiotic activity of inulin, *Agave tequilana* fructan and *Halomonas* levan were investigated by Arrizon et al [86]. Growth of *Lactobacillus* species was observed in all fructans. On the other hand, inulin and levan increased also growth of *Bifidobacteria*. Moreover, growth of pathogenic bacteria (*Salmonella typhimurium*, *Listeria monocytogenes* and *Clostridium spp.*) decreased by inulin and *Halomonas* levan while *Agave tequilana* fructan had no effect. Adamberg et al. [87] studied effects of levan on gut microbiota and enriched *Bacterioides*, *Escherichia*, *Streptococus* and *Faecalibacterium* is observed. Mardo et al. [88] investigated biochemical properties of endolevanase BT1760 on six bacterial levan synthesized by levan sucrase Lsc3 of *Pseudomonas syringae pv*. Tomato, mutant Asp300Asn mutant type, levan from *Zymomonas mobilis*, *Ervinia herbicola* and *Halomonas smyrnensis* levan and timothy grass isolated levan degradation into fructooligosaccharides. BT1760 degraded levans into FOS with DP 2 to 13. All levans were hydrolyzed successfully by BT1760 at body temperature (37°C) and pH between 5-6 and study concluded that plant or bacteria derived levan serve as a prebiotic for *B. thetaiotaomicron* and promote SCFA synthesis by gut microbiota. Enzymatically derived low molecular weight oligosaccharide β-(2-6) (FOS) from levan is investigated as a new carbon source for bifidobacterial growth (*Bifidobacterium adolescentis*, *B. longum*, *B. breve*, and *B. pseudocatenulatum*) by Marx et al [66] and formation of SCFAs were different between species and highest SCFA production is observed in *B. adolescentis*. Zhao et al [89] investigated effect of levan on 96 pigs for 6 weeks and concluded that levan decreased *E. coli* count and increased growth of *Lactobacillus.*

5.2 Immunity and gut barrier function

Barrier function of gut is important for health of host and generally maintained by epithelial barrier. Corruption of epithelial barrier or failure in barrier function may result with several diseases like pathogen infection, obesity, necrotizing enterocolitis, irritable bowel syndrome, inflammatory bowel disease and diabetes [90]. Prebiotics effect host immunity via interaction with immune system cells. Various surface receptors of immune system cells like T and B lymphocytes recognize several carbohydrate structures and some of them bind to those structures of fibers. Neutrophils, macrophages, and dendritic cells have several carbohydrate receptors like Ca^{+} dependent mannose receptor and langerin and Ca^{+} independent Dectin 1 and Dectin 2. Most of those receptors belong to C type lectin (CLR) family that have conserved residues for recognition of carbohydrates [47]. β-glucan groups of carbohydrates have ability to bind directly to macrophage, monocyte, neutrophil and dendritic cell receptors [37] Dectin-1 receptor binds β-glucan structures of fibers and stimulate immune response via cytokine secretion from dendritic cells and stimulates secretion of IL-2, IL6, IL-10, IL-12 and TNF-α [37, 91, 92]. Dectin-1 also play role in TLR related inflammatory signal production via inducing inflammatory gene expression through protein kinase and phosphatase signaling [47]. Endocytosis and phagocytosis is initiated by activation of Dectin 1 [37]. Prebiotic and antipathogenic activities of levan was studied by Yang et al [93, 94] and they reported that enterotoxigenic *E.coli* adhesion to the mucosa is decreased while growth of

beneficial bacterial populations was increased. Fooks and Gibson [95] searched effect of several prebiotics on probiotic and pathogen bacteria and indicated that FOS, inulin, XOS and their mixture inhibited pathogens (*E.coli*, *C. jejuni* and *S. enteridis*) greater than lactulose, lactitol, starch and dextran. It is reported that β-(2-1)-fructans improve intestinal epithelial cell barrier function through Toll-like-2 (TLR-2) receptors. TLR activity and cytokine production is related with chain length of β-(2-1)-fructans and short chain β-(2-1)-fructans are reported to induce regulatory cytokine secretion (IL-10 / IL-12 ratio) more compared to long chain β-(2-1)-fructans [96]. Vogt et al. [96] studied effect of different chain length β-(2-1)-fructans on T84 human intestinal epithelial cell line *in-vitro* and found that time and chain length dependent cell barrier protective effect is maintained on T84 cells via TLR-2 and this showed that β-(2-)1-fructans might target mainly TLR-2 receptor and less TLR-4, 5, 6, 7, and 8 on epithelial cells that result in NF-κB/AP-1 activation to improve host health on TLR-2 dependent mechanisms. Fransen et al. [97] investigated effect of short and long chain β-(2-1) fructans (Frutalose® OFP and Frutafit® TEX) on conventional and germ free mice for 5 days. .Long and short chain fructans increased T-helper cells, enhanced 2-alpha-l-fucosyltransferase 2 expression and IL- 22 dependent genes in conventional mice ileum. Long chain fructans affect B cell response in germ free mice while short chain fuctans increased T regulatory and dendritic cells. Enhanced immunity is observed in germ free mice thus study concluded that immunity is partially dependent to microbiota in a chain length independent manner. Li and Kim [98] investigated effect of levan-type fructan (0, 0.05, 0.1 and 0.2%) on growth, blood profile and fecal microbiota on 80 growing pigs for 42 days. *Lactobacillus* is increased in tract while weight gain is also improved. *E. coli* LPS injection induced blood lymphocyte, serum cortisol level, IL-6, TNF-α increase and levan-type fructan fed pigs showed decrease in these levels at the time dependent interval. Study concluded that 0.1 % levan type fructan supplementation can enhance growth, digestibility, fecal *Lactobacillus* count and immune response in inflammation. Effect of inulin and short chain FOS on gut epithelial barrier function was investigated by Wu et al. [99] *in- vitro*. They concluded that barrier function enhancement can be maintained by activating host epithelial cell signaling through protein kinase C (PKC) δ-dependent mechanism and tight junction induction. Effect of inulin type fructan (DP 10-60, DP 25) on immunity was investigated by Voght et al [100] *in- vitro* and *in- vivo* with 40 heathy human subjects vaccinated against hepatitis B. Development of Anti-HbsAg-titer and lymphocyte are investigated. Th-1 cell increase and TLR-2 stimulation is observed for inulin type fructans (DP 10-60) better than DP 2-25 in *in- vitro* studies. DP 10- 60 increased anti- HBsAg titer, Th-1 cell and transitional B cells. Both fructans increased cytokine production and NK- cells time dependent. Results concluded immunity against pathogenic hepB epitopes was only supported by long chain fructans. Thick mucus layer of gut epithelium with digestive enzymes, antimicrobial peptides and immunoglobulins protect host from pathogens [37]. It was reported that gel- like chemical barriers formed by higher amount of mucin inhibit translocation or colonization of pathogens such *Salmonella* spp., *Shigella* spp. *Vibrio cholerae* and *E.coli* [37]. Acetate protects organism from lethal infections through epithelial cell defense by binding G protein couples receptors on immune cells and regulate immune response [37]. Butyrate is known to induce expression of antimicrobial peptide LL37 that prevent bacterial infections while supplying energy for colon epithelium and increasing proliferation of those cells during injury and reduce colonic inflammation [37, 101, 102]. Rodriguez et al. [103] investigated symbiotic effect of FOS (Beneo®-95) and resistant starch (RS) (Fibersol®-2) on healthy and trinitrobenzenesulphonic acid-(TNBS) colitic rats and found that symbiotic consumption increased *Lactobacilli* and *Bifidobacteria* furthermore increase expression of MUC2 in colon and TNBS models anti-inflammatory activity in the colon.

6. Fructans rich diet in disease

6.1 Colorectal Cancer

Consumption of prebiotics like β-glucans, dietary fibers, fructans and resistant starch play important role in prevention of colorectal cancer through production of SCFA (acetate, butyrate, and propionate), modulation of gene expression in tumor cells, decreasing activity of cancer triggering bacteria, action as diluting agent to reduce interaction of mutagens and carcinogens with epithelial cells. Production of SCFAs decrease pH in the colon which result in increased mineral solubility like calcium, magnesium, and iron. By the increase of calcium absorption pathogen growth can be prevented. Increased calcium absorption also reduces the risk of osteoporosis via bone calcium depletion [37]. Butyrate induces cell apoptosis, differentiation and reduces proliferation of malignant cells through initiation of cell cycle arrests at phase G_1. It also stimulates protein synthesis like alkaline phosphatase, hormone receptors and glycoproteins and act on colonocytes to reduce colorectal cancer [37, 45]. Bolognani et al. [104] investigated effects of oligosaccharides and lactic acid producing bacteria on early neoplasia precursor aberrant crypt foci (ACF) induction by carcinogens on ACF induced rats. *L. acidophilus* or inulin fed rats showed significant ACF decrease and protection from colorectal cancer. Taper and Roberfroid [105] investigated role of inulin and oligofructose (15%) on transplantable mouse tumor and showed that tumor growth is inhibited by supplementation of inulin and oligofructose. Synergy 1® (chicory inulin) and Metlin® (Mexican agave inulin) are tested against colon cancer and bone calcium metabolism in mice and rats with two different models by Riviera- Huerta et al. [106]. Results indicated that inulin inhibited dextran sulfate sodium induced colitis and colon cancer development in mice through reducing concentration of TNF-α (tumor necrosis factor alpha).

Formation of polyps and villous atrophy and lymphoid hyperplasia are prevented. Studies with rats showed increase of bone densitometry in femur and vertebra.

6.2 Irritable Bowel Syndrome and Inflammatory Bowel Disease

Irritable Bowel Syndrome (IBS) is a gastrointestinal disorder which results in abdominal pain, discomfort and change in stool characteristics and frequency. IBS is related with gut barrier function [90]. Butyrate is known to have positive effect on reducing symptoms of IBS [107] and improving gut barrier function via epithelial proliferation. Symbiotic approach to reduce symptoms and improve health of IBS patients via increasing production of lactic acid and butyrate is reported by various studies [90]. Beneficial effect of inulin-type fructans and FOS on IBS disease is reported [108]. Inflammatory Bowel Disease (IBD) is the general name of two inflammatory gut related diseases; Chron's disease (CD) and Ulcerative colitis (UC) and mucosal inflammation and impaired barrier function is observed in IBD patients [109] CD is a transmural mononuclear inflammation and generally effect colon and terminal ileum while UC only effect colon. Loss of goblet cells, epithelial cell damage and neutrophil infiltration occurs in UC [90]. Gut microbiota, barrier function of gut and mucus secretion play important roles in reducing symptoms of IBD thus prebiotic support to improve gut microbiota may be beneficial in reducing IBD symptoms while improving quality of life in those patients. *Lactobacillus* GG is reported beneficial in Children Chron's disease by improving gut barrier function [110]. Paineau et al [111] investigated effect of short chain fructooligosaccharides (sc-FOS) on patients with functional bowel disorders and improved daily activity, and life quality is reported thus, consumption of FOS may improve digestive comfort due to their prebiotic activities. Ulisse et al [112] investigated effect of probiotic mixture VSL#3 on biopsy specimens of pouchitis patients and cytokine secretion (IL-10) was increased. Cox et al investigated [113] effect of fermentable carbohydrates on functional gastrointestinal symptoms (FGS) in IBD. Patients with IBD and FGS get fructan (12g/day), Galactooligosachharide (GOS) (6 g/day), Sorbitol (6 g/day) and glucose placebo (12g/day). Fewer patients reported relief of FGS in fructan diet while GOS and sorbitol exacerbated symptoms of FGS in IBD.

6.3 Metabolic Syndrome, Diabetes, Obesity, and Cardiovascular Diseases

Diabetes, obesity, cardiovascular diseases, and metabolic syndrome are closely related and recently increasing disorders and one can trigger another. Side effects of drugs and increased popularity on prebiotic consumption as a treatment for such diseases made researchers investigate effects of prebiotics in disease and their interaction with host and microbiota. Glucose intolerance, insulin resistance, hyperinsulinemia, hypertension, dyslipidemia and impaired fasting glycemia together with obesity refers to metabolic syndrome [114]. It is reported that metabolic syndrome have risk for development of type 2 diabetes, atherosclerosis and hypertension [115]. Development of metabolic syndrome could be reduced by consumption of prebiotics (oligosaccharides) to improve gut microbiota, gut barrier function and reducing oxidative stress [114]. Diabetes mellitus (DM) is an insulin metabolism related disease where body cannot use or produce insulin effectively (type 1 and type 2) [116]. Diabetes may result in dementia, kidney and cardiovascular diseases, increased mortality and morbidity , hypertension and steatosis hepatis and cancer [117].

Risk of heart diseases can be increased with plasma LDL cholesterol levels and dietary content can reduce plasma cholesterol levels via production of SCFAs. Especially propionate inhibits cholesterol synthesizing enzymes, arrange distribution of cholesterol from plasma to liver, improve secretion of bile acids [37, 118]. Lowering blood lipid and cholesterol levels, reduction of obesity and diabetes and improving absorption of minerals could reduce the risk of hypertension and hearth diseases [108].

Obesity is an impaired lipid metabolism related disease and increase in serum triglycerides and cholesterol is observed [119]. It is known hat obesity can trigger hypertension [120], myocardial infarct [121] and diabetes [122]. Gut microbiota play crucial role on obesity and related metabolic disorders. Nutrition is important for establishment of this microbiota. Indirect effect of colonic fermentation is on the pancreas and adipose tissue hormones which have role energy metabolism thus, colonic fermentation, microbiota and prebiotics are associated with obesity [45, 123].

Effect of inulin type fructans (oligofructose and inulin) on metabolic syndrome is investigated by Rault-Nania et al [124] on fructose fed metabolic syndrome rat model for 4 weeks. Inulin type fructan supplemented diet prevented induction of high blood pressure, heart peroxidation susceptibility and renal damages and hypertriglyceridemia. Increased levels of oxidative stress and lowered AMPK activity is also observed which promote metabolic syndrome. Parnell et al [125] investigated effects of oligofructose supplementation on 37 obese subjects (BMI 30.4 kg/m^2) for 12 weeks. Study concluded that obesity related inflammation markers might be mitigated by oligofructose supplementation via reducing PAI-1, risk factor for thrombosis, and metabolic endotoxemia through reducing plasma lipopolysaccharide levels. Effect of oligofructose enriched inulin (8 g/day) on body composition, markers of inflammation, fecal bile acid levels and microbiota composition is investigated by Nicoloucci et al.[126] on obese children for 16 weeks. Decreased body weight, percent body fat, percent trunk fat, reduction in IL-6, serum triglycerides is observed. *Bifidobacterium* and *Bacteroides vulgatus* increased in fecal samples and bile acid concentration if fecal samples only in placebo group. Dalzenne et al [127] investigated metabolism on FOS (20% of body weight) and inulin (10% body weight) fed rats for 30 days. Decreased serum and liver triglyceride level is observed in FOS while no change on cholesterol levels. Only the ratio of HDL and LDL cholesterol is increased. Seric and hepatic lipid modifications observed both groups. Kok et

al. [128] investigated effect of fructooligosaccharides (Raftilose ®) (100g/kg) on rats for 30 days and found that FOSs reduced de-novo fatty acid synthesis in liver and lowered serum insulin levels. Propionate inhibits de-novo synthesis of fatty acids, hepatic gluconeogenesis in hepatocytes, suppress plasma triacylglycerol, control blood glucose level that play role in insulin resistance, and show anti-inflammatory effects [45, 129]. Patent application of Haber et al [130] claimed role of levan in treatment for hyperlipidemia and hypercholesterolemia and as an arteriosclerosis reducing agent. Yamamoto et al [131] studied 1-5% high molecular levan containing cholesterol free diet fed rats for 1 month and results showed increased fecal sterol and lipid excretion which may conclude sterol absorption preventing activity of levan while decreased serum cholesterol level is also observed. Regulation of appetite through hormones and diet play important role in body weight reduction, blood glucose level regulation and obesity. Appetite can be modulated with prebiotic supplementation by increasing plasma gut peptide concentrations and SCFAs in gut like acetate, butyrate, and propionate. Propionate is known to reduce body weight through stimulation of leptin. SCFAs can suppress appetite through hormonal stimulation like Glucagon like peptide- 1 (GLP-1) and Peptide YY in gut lumen secreted by L colonocytes [46]. Appetite regulation of acetate through parasympathetic nervous system and stimulated insulin, leptin and ghrelin secretion is reported by Perry et al [132]. A study about appetite, inulin and FOS relationship is reported by Cani et al [133]. 10 healthy adults received 16 grams of prebiotics for 2 weeks and results showed that lowered hunger and appetite is observed. Plasma GLP-1level increased while postprandial plasma glucose response is decreased. Cani et al [134] investigated effect of oligofructose (Orafti, Belgium) on obese mice and results showed that prebiotic modulation increased *bifidobacteria*, and increased gut barrier function via GLP-2. Effect of oligofructose (Orafti, Belgium) on high fat diet fed mice is also investigated by Cani et al [135]. It is concluded that oligofructose uptake reduces the development of diabetes and obesity via glucagon like 1 receptor. GLP-1 receptor play important role in decreasing blood glucose level and steatosis in obesity and diabetes [136]. Kang et al [137] studied effect of *Zymomonas* levan on obese and hyperlipidemia suppressed lab rats. Reduced daily weight gain even on high fat diet fed rats is observed. Hyperinsulinemia, hyperglycemia, levels of free fatty acids serum triglycerides, serum cholesterol, unilocular fat tissue improvement and adipocyte hypertrophy is reduced in dose depending manner. Parnel et al. [138] investigated effect of fructooligosaccharides (21g/day) for 22 weeks in obese adults and enhanced weight loss and improved blood glucose level is observed. Oh and Lee et al [139] searched anti-obesity activity of fermented red ginseng, levan and their combination for 11 weeks on high fat diet fed mice. Combination of levan and red fermented ginseng consuming group showed decreased body weight gain, white adipose tissue weight, insulin resistance, leptin, and fasting blood glucose level. Energy intake and appetite control in oligofructose enriched inulin supplementation (8 g/day) is investigated by Hume et al [140] in 42 overweight and obese children (7-12 y) for 16 weeks. Feeling of fullness, fasting adiponectin and ghrelin increased in prebiotic consumption while prospective food consumption at breakfast buffet, energy intake and BMI is decreased.

Kazak et al. [141] investigated antidiabetic activity of *Halomonas* levan on pancreatic INS-1E pancreatic cell line *in-vitro*. Decreased reactive oxygen species (ROS) generation and apoptosis is observed. *Bacillus* levan containing diet decreased plasma glucose and serum lipid levels and increased glycogen level on diabetic rats [142]. Gao et al [143] studied effect of butyric acid (5% wt/wt) on dietary-obese high fat diet fed mice and found that prevention of insulin resistance formation is prevented by butyrate while fatty acid oxidation and thermogenesis, mitochondrial function and biogenesis are enhanced and adiposity is reduced. Another study about HDL cholesterol increasing and LDL cholesterol lowering effect of *Bacillus* levan on cholesterol rich diet fed diabetic rats for 2 months is reported by Belghith et al [144]. Gobinath et al [145] investigated antidiabetic effect of xylooligosachharides and fructooligosaccharides (10% w/w) on diabetes induced rats for 6 weeks. Hyperglycemia and cholesterol levels are reduced and body weight is improved and *Bifidobacteria* and *Lactobacillus* is increased in both samples. Activity of antioxidant enzymes (catalase and glutathione reductase) increased in the blood of diabetic rats. Glycation end products, glycosuria, proteinuria, diabetic neuropathy and concentration of blood creatinine and urea reduced. Chen et al. [146] investigated effect of inulin type fructans; (DP 2-25 and DP 10-60) on gut barrier function related type 1 diabetes (TD1) in nonobese diabetic rats. Long chain fructans reduced the incidence of TD1and increased $CD25^+$, $FoxP3^+$, $CD4^+$ and decreased IL-17A, $CD4^+$, Th 17 cells, cytokine production in pancreas, spleen and colon is observed. Fructans also enhanced tight junction proteins ocludin, claudin-2, antimicrobial peptides defensin-1 and cathelicidin related peptide production. Result concluded that long chain fructans delay development of diabetes via modulation of gut and pancreatic immunity, barrier function and microbiota. Oligofurctose containing a yogurt drink (Orafti® P95) and inulin containing fruit jelly (Orafti® GR) is investigated on 40 -42 healthy adults by Lightowler et al. [147] and Inulin consumption lowered glycemic response in higher amount than Oligofructose consumption. In both studies insulin response is lowered. Study concluded that substitution of glycemic sugar by inulin or oligofructose reduce blood glucose response to foods.

7. Conclusions and future perspectives

The complex and diverse gut microbial communities play crucial roles in human health due to their metabolic, immunologic, and protective properties in a number of diseases. To improve bacterial community and their functions,

dietary approach play significant role. The most important dietary strategy is to modulate metabolic function of microbiota via consumption of dietary fibers and prebiotics [148]. Carbohydrate polymers are called dietary fibers or prebiotics if they cannot be digested by human alimentary enzymes and absorbed in colon. Therefore these carbohydrates are selectively fermented in colon by gut microbiota and generate short chain fatty acids (SCFAs) like butyrate, acetate and propionate [35, 149]. Among prebiotics most known group is fructans that are found commonly in nature. Recent studies put forth that use of fructans as prebiotic is beneficial to human health and may counteract the development of various diseases such as metabolic syndrome, diabetes, obesity, cardiovascular diseases, colorectal cancer, irritable bowel syndrome and inflammatory bowel disease. Although fructans are valuable functional food ingredients, they requires further research. In further studies, derivatives of fructans and their combinations both with each other and with natural fructans can be investigated for their healing effects in various diseases.

Acknowledgements The support by 115O495 TUBITAK and Fen-A1305150178 BAP projects are gratefully acknowledged.

References

[1] Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. 2006;124(4):837-48.
[2] Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human–microbe mutualism and disease. Nature. 2007;449(7164):811-8.
[3] Whitman WB, Coleman DC, Wiebe WJ. Prokaryotes: the unseen majority. Proceedings of the National Academy of Sciences. 1998;95(12):6578-83.
[4] Haynes M, Rohwer F. The human virome. Metagenomics of the human body: Springer; 2011. p. 63-77.
[5] Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett C, Knight R, Gordon JI. The human microbiome project: exploring the microbial part of ourselves in a changing world. Nature. 2007;449(7164):804.
[6] Ursell LK, Metcalf JL, Parfrey LW, Knight R. Defining the human microbiome. Nutrition reviews. 2012;70(suppl 1):S38-S44.
[7] Shendure J, Ji H. Next-generation DNA sequencing. Nature biotechnology. 2008;26(10):1135-45.
[8] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nature methods. 2010;7(5):335-6.
[9] Hamady M, Walker JJ, Harris JK, Gold NJ, Knight R. Error-correcting barcoded primers allow hundreds of samples to be pyrosequenced in multiplex. Nature methods. 2008;5(3):235.
[10] O'Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO reports. 2006;7(7):688-93.
[11] Gill SR, Pop M, DeBoy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. science. 2006;312(5778):1355-9.
[12] Chow J, Lee SM, Shen Y, Khosravi A, Mazmanian SK. Host–bacterial symbiosis in health and disease. Advances in immunology. 2010;107:243.
[13] Hull MW, Chow AW. Indigenous microflora and innate immunity of the head and neck. Infectious disease clinics of North America. 2007;21(2):265-82.
[14] Neish AS. Microbes in gastrointestinal health and disease. Gastroenterology. 2009;136(1):65-80.
[15] Verstraelen H. Cutting edge: the vaginal microflora and bacterial vaginosis. Verhandelingen-Koninklijke Academie voor Geneeskunde van Belgie. 2007;70(3):147-74.
[16] Gebbers J, Laissue J. Immunologic structures and functions of the gut. Schweizer Archiv fur Tierheilkunde. 1988;131(5):221-38.
[17] Gordon JH, Dubos R. The anaerobic bacterial flora of the mouse cecum. Journal of Experimental Medicine. 1970;132(2):251-60.
[18] Harris MA, Reddy CA, Carter GR. Anaerobic bacteria from the large intestine of mice. Applied and environmental microbiology. 1976;31(6):907-12.
[19] SAVAGE DC. Associations of indigenous microorganisms with gastrointestinal mucosal epithelia. The American journal of clinical nutrition. 1970;23(11):1495-501.
[20] Mirzaei MK, Maurice CF. Menage a trois in the human gut: interactions between host, bacteria and phages. Nature Reviews Microbiology. 2017;15(7):397-408.
[21] Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. science. 2005;308(5728):1635-8.
[22] Frank DN, Amand ALS, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proceedings of the National Academy of Sciences. 2007;104(34):13780-5.
[23] Swidsinski A, Loening-Baucke V, Lochs H, Hale LP. Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice. World journal of gastroenterology: WJG. 2005;11(8):1131.
[24] Guarner F, Malagelada J-R. Gut flora in health and disease. The Lancet. 2003;361(9356):512-9.
[25] Fu J, Bonder MJ, Cenit MC, Tigchelaar E, Maatman A, Dekens JA, et al. The gut microbiome contributes to a substantial proportion of the variation in blood lipids. Circulation research. 2015:CIRCRESAHA. 115.306807.
[26] Cook RL, Sobel JD. Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. Review of infectious Diseases. 1990;12(5):856-72.
[27] Mändar R, Mikelsaar M. Transmission of mother's microflora to the newborn at birth. Neonatology. 1996;69(1):30-5.
[28] Huurre A, Kalliomäki M, Rautava S, Rinne M, Salminen S, Isolauri E. Mode of delivery–effects on gut microbiota and humoral immunity. Neonatology. 2008;93(4):236-40.

[29] Mackie RI, Sghir A, Gaskins HR. Developmental microbial ecology of the neonatal gastrointestinal tract. The American journal of clinical nutrition. 1999;69(5):1035s-45s.
[30] Zhang C, Zhang M, Wang S, Han R, Cao Y, Hua W, et al. Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. The ISME journal. 2010;4(2):232-41.
[31] Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell host & microbe. 2008;3(4):213-23.
[32] Sonnenburg JL, Bäckhed F. Diet-microbiota interactions as moderators of human metabolism. Nature. 2016;535(7610):56-64.
[33] Meeting JFWECoFA, Organization WH. Evaluation of Certain Food Additives: Seventy-first Report of the Joint FAO/WHO Expert Committee on Food Additives: World Health Organization; 2010.
[34] Blatchford P, Ansell J, de Godoy M, Fahey G, Garcia-Mazcorro J, Gibson G, et al. Prebiotic mechanisms, functions and applications-A review. International Journal of Probiotics & Prebiotics. 2013;8(4):109.
[35] Slavin J. Fiber and prebiotics: mechanisms and health benefits. Nutrients. 2013;5(4):1417-35.
[36] Cantarel BL, Lombard V, Henrissat B. Complex carbohydrate utilization by the healthy human microbiome. PloS one. 2012;7(6):e28742.
[37] Di Bartolomeo F, Startek J, Van den Ende W. Prebiotics to fight diseases: reality or fiction? Phytotherapy Research. 2013;27(10):1457-73.
[38] Di Bartolomeo F, Van den Ende W. Fructose and fructans: opposite effects on health? Plant foods for human nutrition. 2015;70(3):227-37.
[39] Van den Ende W, El-Esawe SK. Sucrose signaling pathways leading to fructan and anthocyanin accumulation: A dual function in abiotic and biotic stress responses? Environmental and Experimental Botany. 2014;108:4-13.
[40] Peshev D, Van den Ende W. Fructans: prebiotics and immunomodulators. Journal of Functional Foods. 2014;8:348-57.
[41] Saad N, Delattre C, Urdaci M, Schmitter J-M, Bressollier P. An overview of the last advances in probiotic and prebiotic field. LWT-Food Science and Technology. 2013;50(1):1-16.
[42] Hamaker BR, Tuncil YE. A perspective on the complexity of dietary fiber structures and their potential effect on the gut microbiota. Journal of molecular biology. 2014;426(23):3838-50.
[43] Sonnenburg ED, Zheng H, Joglekar P, Higginbottom SK, Firbank SJ, Bolam DN, et al. Specificity of polysaccharide use in intestinal bacteroides species determines diet-induced microbiota alterations. Cell. 2010;141(7):1241-52.
[44] Roy CC, Kien CL, Bouthillier L, Levy E. Short-chain fatty acids: ready for prime time? Nutrition in clinical practice. 2006;21(4):351-66.
[45] Lovegrove A, Edwards C, De Noni I, Patel H, El S, Grassby T, et al. Role of polysaccharides in food, digestion, and health. Critical reviews in food science and nutrition. 2017;57(2):237-53.
[46] Kumari M, Kozyrskyj A. Gut microbial metabolism defines host metabolism: an emerging perspective in obesity and allergic inflammation. Obesity Reviews. 2017;18(1):18-31.
[47] Venema K, Do Carmo AP. Probiotics and prebiotics: Caister Academic Press; 2015.
[48] Le Blay G, Michel C, Blottière HM, Cherbut C. Prolonged intake of fructo-oligosaccharides induces a short-term elevation of lactic acid-producing bacteria and a persistent increase in cecal butyrate in rats. The Journal of nutrition. 1999;129(12):2231-5.
[49] Roberfroid M, Slavin J. Nondigestible oligosaccharides. Critical Reviews in Food Science and Nutrition. 2000;40(6):461-80.
[50] Roberfroid M. Prebiotics: the concept revisited. The Journal of nutrition. 2007;137(3):830S-7S.
[51] Holscher HD, Doligale JL, Bauer LL, Gourineni V, Pelkman CL, Fahey GC, et al. Gastrointestinal tolerance and utilization of agave inulin by healthy adults. Food & function. 2014;5(6):1142-9.
[52] Paseephol T, Sherkat F. Probiotic stability of yoghurts containing Jerusalem artichoke inulins during refrigerated storage. Journal of Functional Foods. 2009;1(3):311-8.
[53] THAMER KG, PENNA AL. Efeito do teor de soro, açúcar e de frutooligossacarídeos sobre a população de bactérias lácticas probióticas em bebidas fermentadas. Revista Brasileira de Ciências Farmacêuticas, São Paulo. 2005;41(3).
[54] van den Heuvel EG, Muijs T, Brouns F, Hendriks HF. Short-chain fructo-oligosaccharides improve magnesium absorption in adolescent girls with a low calcium intake. Nutrition research. 2009;29(4):229-37.
[55] Roberfroid MB. Introducing inulin-type fructans. British Journal of Nutrition. 2005;93(S1):S13-S25.
[56] Yun JW. Fructooligosaccharides—occurrence, preparation, and application. Enzyme and microbial technology. 1996;19(2):107-17.
[57] Singh RS, Singh RP. Production of fructooligosaccharides from inulin by endoinulinases and their prebiotic potential. Food Technology and Biotechnology. 2010;48(4):435.
[58] Ghazi I, Fernandez-Arrojo L, Garcia-Arellano H, Ferrer M, Ballesteros A, Plou FJ. Purification and kinetic characterization of a fructosyltransferase from Aspergillus aculeatus. Journal of Biotechnology. 2007;128(1):204-11.
[59] Wack M, Blaschek W. Determination of the structure and degree of polymerisation of fructans from Echinacea purpurea roots. Carbohydrate research. 2006;341(9):1147-53.
[60] Rivero-Urgell M, Santamaria-Orleans A. Oligosaccharides: application in infant food. Early Human Development. 2001;65:S43-S52.
[61] Sabater-Molina M, Larqué E, Torrella F, Zamora S. Dietary fructooligosaccharides and potential benefits on health. Journal of physiology and biochemistry. 2009;65(3):315-28.
[62] Vega R, Zuniga-Hansen M. The effect of processing conditions on the stability of fructooligosaccharides in acidic food products. Food chemistry. 2015;173:784-9.
[63] Lorenzoni AS, Aydos LF, Klein MP, Rodrigues RC, Hertz PF. Fructooligosaccharides synthesis by highly stable immobilized β-fructofuranosidase from Aspergillus aculeatus. Carbohydrate polymers. 2014;103:193-7.
[64] Charalampopoulos D, Rastall RA. Prebiotics in foods. Current opinion in biotechnology. 2012;23(2):187-91.
[65] Selak M, Rivière A, Moens F, Van den Abbeele P, Geirnaert A, Rogelj I, et al. Inulin-type fructan fermentation by bifidobacteria depends on the strain rather than the species and region in the human intestine. Applied microbiology and biotechnology. 2016;100(9):4097-107.

[66] Marx SP, Winkler S, Hartmeier W. Metabolization of β-(2, 6)-linked fructose-oligosaccharides by different bifidobacteria. FEMS Microbiology Letters. 2000;182(1):163-9.
[67] Pinelo M, Jonsson G, Meyer AS. Membrane technology for purification of enzymatically produced oligosaccharides: molecular and operational features affecting performance. Separation and Purification Technology. 2009;70(1):1-11.
[68] Jakob F, Pfaff A, Novoa-Carballal R, Rübsam H, Becker T, Vogel RF. Structural analysis of fructans produced by acetic acid bacteria reveals a relation to hydrocolloid function. Carbohydrate polymers. 2013;92(2):1234-42.
[69] Yen C-H, Kuo Y-W, Tseng Y-H, Lee M-C, Chen H-L. Beneficial effects of fructo-oligosaccharides supplementation on fecal bifidobacteria and index of peroxidation status in constipated nursing-home residents—A placebo-controlled, diet-controlled trial. Nutrition. 2011;27(3):323-8.
[70] Jakobsdottir G, Nyman M, Fåk F. Designing future prebiotic fiber to target metabolic syndrome. Nutrition. 2014;30(5):497-502.
[71] Delgado GTC, Tamashiro WM, Pastore GM. Immunomodulatory effects of fructans. Food Research International. 2010;43(5):1231-6.
[72] Licht TR, Ebersbach T, Frøkiær H. Prebiotics for prevention of gut infections. Trends in food science & technology. 2012;23(2):70-82.
[73] Kang SA, Jang K-H, Seo J-W, Kim KH, Kim YH, Rairakhwada D, et al. Levan: applications and perspectives. Microbial production of biopolymers and polymer precursors Caister Academic Press, Norwich. 2009:145-61.
[74] Öner ET, Hernández L, Combie J. Review of levan polysaccharide: From a century of past experiences to future prospects. Biotechnology advances. 2016;34(5):827-44.
[75] Erginer M, Akcay A, Coskunkan B, Morova T, Rende D, Bucak S, et al. Sulfated levan from Halomonas smyrnensis as a bioactive, heparin-mimetic glycan for cardiac tissue engineering applications. Carbohydrate polymers. 2016;149:289-96.
[76] Kang T, Jung S, Kang S, Jang K, Jang E, Kim S, et al. Preparation of levan oligosaccharides by acid hydrolysis and its application in growth of lactic acid-producing bacteria. Korean J Biotechnol Bioeng. 2002;17:137-41.
[77] Samal L, Chaturvedi V, Pattanaik A. Effects of dietary supplementation with Jerusalem artichoke (Helianthus tuberosus L.) tubers on growth performance, nutrient digestibility as well as activity and composition of large intestinal microbiota in rats. J Anim Feed Sci. 2017;26(1):50-8.
[78] Chung WSF, Walker AW, Louis P, Parkhill J, Vermeiren J, Bosscher D, et al. Modulation of the human gut microbiota by dietary fibres occurs at the species level. BMC biology. 2016;14(1):3.
[79] Holscher HD, Bauer LL, Gourineni V, Pelkman CL, Fahey GC, Swanson KS. Agave inulin supplementation affects the fecal microbiota of healthy adults participating in a randomized, double-blind, placebo-controlled, crossover trial. The Journal of nutrition. 2015;145(9):2025-32.
[80] Majid HA, Cole J, Emery PW, Whelan K. Additional oligofructose/inulin does not increase faecal bifidobacteria in critically ill patients receiving enteral nutrition: a randomised controlled trial. Clinical Nutrition. 2014;33(6):966-72.
[81] Silva P, Garcia S, Baldo C, Celligoi M. Prebiotic activity of fructooligosaccharides produced by Bacillus subtilis natto CCT 7712. Acta Alimentaria. 2017;46(2):145-51.
[82] Martinez-Gutierrez F, Ratering S, Juárez-Flores B, Godinez-Hernandez C, Geissler-Plaum R, Prell F, et al. Potential use of Agave salmiana as a prebiotic that stimulates the growth of probiotic bacteria. LWT-Food Science and Technology. 2017.
[83] Porras-Domínguez JR, Ávila-Fernández Á, Rodríguez-Alegría ME, Miranda-Molina A, Escalante A, González-Cervantes R, et al. Levan-type FOS production using a Bacillus licheniformis endolevanase. Process Biochemistry. 2014;49(5):783-90.
[84] Korakli M, Gänzle M, Vogel R. Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by Lactobacillus sanfranciscensis. Journal of applied microbiology. 2002;92(5):958-65.
[85] Dal Bello F, Walter J, Hertel C, Hammes WP. In vitro study of prebiotic properties of levan-type exopolysaccharides from lactobacilli and non-digestible carbohydrates using denaturing gradient gel electrophoresis. Systematic and Applied Microbiology. 2001;24(2):232-7.
[86] Arrizon J, Hernández-Moedano A, Oner ET, González-Avila M. In vitro prebiotic activity of fructans with different fructosyl linkage for symbiotics elaboration. International Journal of Probiotics & Prebiotics. 2014;9(3):69.
[87] Adamberg K, Tomson K, Talve T, Pudova K, Puurand M, Visnapuu T, et al. Levan enhances associated growth of Bacteroides, Escherichia, Streptococcus and Faecalibacterium in fecal microbiota. PloS one. 2015;10(12):e0144042.
[88] Mardo K, Visnapuu T, Vija H, Aasamets A, Viigand K, Alamäe T. A Highly Active Endo-Levanase BT1760 of a Dominant Mammalian Gut Commensal Bacteroides thetaiotaomicron Cleaves Not Only Various Bacterial Levans, but Also Levan of Timothy Grass. PloS one. 2017;12(1):e0169989.
[89] Zhao P, Wang J, Kim I. Evaluation of dietary fructan supplementation on growth performance, nutrient digestibility, meat quality, fecal microbial flora, and fecal noxious gas emission in finishing pigs. Journal of animal science. 2013;91(11):5280-6.
[90] Bron PA, Kleerebezem M, Brummer R-J, Cani PD, Mercenier A, MacDonald TT, et al. Can probiotics modulate human disease by impacting intestinal barrier function? British Journal of Nutrition. 2017;117(1):93-107.
[91] Wismar R, Brix S, Frøkiær H, Laerke HN. Dietary fibers as immunoregulatory compounds in health and disease. Annals of the New York Academy of Sciences. 2010;1190(1):70-85.
[92] Yamasaki S, Matsumoto M, Takeuchi O, Matsuzawa T, Ishikawa E, Sakuma M, et al. C-type lectin Mincle is an activating receptor for pathogenic fungus, Malassezia. Proceedings of the National Academy of Sciences. 2009;106(6):1897-902.
[93] Yang Y, Galle S, Le MHA, Zijlstra RT, Gänzle MG. Feed fermentation with reuteran-and levan-producing Lactobacillus reuteri reduces colonization of weanling pigs by enterotoxigenic Escherichia coli. Applied and environmental microbiology. 2015;81(17):5743-52.
[94] Yang Y, Zhao X, Le MH, Zijlstra RT, Gänzle MG. Reutericyclin producing Lactobacillus reuteri modulates development of fecal microbiota in weanling pigs. Frontiers in microbiology. 2015;6.
[95] Fooks LJ, Gibson GR. In vitro investigations of the effect of probiotics and prebiotics on selected human intestinal pathogens. FEMS Microbiology Ecology. 2002;39(1):67-75.

[96] Vogt L, Ramasamy U, Meyer D, Pullens G, Venema K, Faas MM, et al. Immune modulation by different types of β2→ 1-fructans is toll-like receptor dependent. PLoS One. 2013;8(7):e68367.
[97] Fransen F, Sahasrabudhe NM, Elderman M, Bosveld M, Aidy SE, Hugenholtz F, et al. β2→ 1-Fructans Modulate the Immune System In Vivo in a Microbiota-Dependent andn-Independent Fashion. 2017.
[98] Li J, Kim I. Effects of levan-type fructan supplementation on growth performance, digestibility, blood profile, fecal microbiota, and immune responses after lipopolysaccharide challenge in growing pigs. Journal of animal science. 2013;91(11):5336-43.
[99] Wu RY, Abdullah M, Määttänen P, Pilar AVC, Scruten E, Johnson-Henry KC, et al. Protein kinase C δ signaling is required for dietary prebiotic-induced strengthening of intestinal epithelial barrier function. Scientific reports. 2017;7:40820.
[100] Vogt LM, Elderman ME, Borghuis T, Haan BJ, Faas MM, Vos P. Chain length-dependent effects of inulin-type fructan dietary fiber on human systemic immune responses against hepatitis-B. Molecular Nutrition & Food Research. 2017.
[101] Ashida H, Ogawa M, Kim M, Mimuro H, Sasakawa C. Bacteria and host interactions in the gut epithelial barrier. Nature chemical biology. 2012;8(1):36-45.
[102] Guilloteau P, Martin L, Eeckhaut V, Ducatelle R, Zabielski R, Van Immerseel F. From the gut to the peripheral tissues: the multiple effects of butyrate. Nutrition research reviews. 2010;23(02):366-84.
[103] Rodríguez-Cabezas ME, Camuesco D, Arribas B, Garrido-Mesa N, Comalada M, Bailón E, et al. The combination of fructooligosaccharides and resistant starch shows prebiotic additive effects in rats. Clinical nutrition. 2010;29(6):832-9.
[104] Bolognani F, Rumney CJ, Pool-Zobel BL, Rowland IR. Effect of lactobacilli, bifidobacteria and inulin on the formation of aberrant crypt foci in rats. European journal of nutrition. 2001;40(6):293-300.
[105] Taper H, Lemort C, Roberfroid M. Inhibition effect of dietary inulin and oligofructose on the growth of transplantable mouse tumor. Anticancer research. 1997;18(6A):4123-6.
[106] Rivera-Huerta M, Lizárraga-Grimes VL, Castro-Torres IG, Tinoco-Méndez M, Macías-Rosales L, Sánchez-Bartéz F, et al. Functional Effects of Prebiotic Fructans in Colon Cancer and Calcium Metabolism in Animal Models. BioMed Research International. 2017;2017.
[107] Vanhoutvin S, Troost F, Kilkens T, Lindsey P, Hamer H, Jonkers D, et al. The effects of butyrate enemas on visceral perception in healthy volunteers. Neurogastroenterology & Motility. 2009;21(9):952-e76.
[108] Yeo S-K, Ooi L-G, Lim T-J, Liong M-T. Antihypertensive properties of plant-based prebiotics. International journal of molecular sciences. 2009;10(8):3517-30.
[109] Pastorelli L, De Salvo C, Mercado JR, Vecchi M, Pizarro TT. Central role of the gut epithelial barrier in the pathogenesis of chronic intestinal inflammation: lessons learned from animal models and human genetics. Frontiers in immunology. 2013;4:280.
[110] Gupta P, Andrew H, Kirschner BS, Guandalini S. Is Lactobacillus GG helpful in children with Crohn's disease? Results of a preliminary, open-label study. Journal of pediatric gastroenterology and nutrition. 2000;31(4):453-7.
[111] Paineau D, Payen F, Panserieu S, Coulombier G, Sobaszek A, Lartigau I, et al. The effects of regular consumption of short-chain fructo-oligosaccharides on digestive comfort of subjects with minor functional bowel disorders. British Journal of Nutrition. 2008;99(02):311-8.
[112] Ulisse S, Gionchetti P, D'Alò S, Russo FP, Pesce I, Ricci G, et al. Expression of cytokines, inducible nitric oxide synthase, and matrix metalloproteinases in pouchitis: effects of probiotic treatment. The American journal of gastroenterology. 2001;96(9):2691-9.
[113] Cox SR, Prince AC, Myers CE, Irving PM, Lindsay JO, Lomer MC, et al. Fermentable carbohydrates (FODMAPs) exacerbate functional gastrointestinal symptoms in patients with inflammatory bowel disease: a randomised, double-blind, placebo-controlled, cross-over, re-challenge trial. Journal of Crohn's and Colitis. 2017:jjx073.
[114] Van den Ende W, Peshev D, De Gara L. Disease prevention by natural antioxidants and prebiotics acting as ROS scavengers in the gastrointestinal tract. Trends in Food Science & Technology. 2011;22(12):689-97.
[115] Agirbasli M, Tanrikulu AM, Berenson GS. Metabolic syndrome: Bridging the gap from childhood to adulthood. Cardiovascular therapeutics. 2016;34(1):30-6.
[116] Wu J, Shi S, Wang H, Wang S. Mechanisms underlying the effect of polysaccharides in the treatment of type 2 diabetes: A review. Carbohydrate polymers. 2016;144:474-94.
[117] Dina C. New insights into the genetics of body weight. Current Opinion in Clinical Nutrition & Metabolic Care. 2008;11(4):378-84.
[118] Arora T, Sharma R, Frost G. Propionate. Anti-obesity and satiety enhancing factor? Appetite. 2011;56(2):511-5.
[119] Kang SA, Hong K, Jang K-H, Kim Y-Y, Choue R, Lim Y. Altered mRNA expression of hepatic lipogenic enzyme and PPARα in rats fed dietary levan from Zymomonas mobilis. The Journal of nutritional biochemistry. 2006;17(6):419-26.
[120] Matsuo T, Sairenchi T, Suzuki K, Tanaka K, Muto T. Long-term stable obesity increases risk of hypertension. International journal of obesity. 2011;35(8):1056-62.
[121] Yusuf S, Hawken S, Ôunpuu S, Bautista L, Franzosi MG, Commerford P, et al. Obesity and the risk of myocardial infarction in 27 000 participants from 52 countries: a case-control study. The Lancet. 2005;366(9497):1640-9.
[122] Bray G. Obesity increases risk for diabetes. International journal of obesity and related metabolic disorders: journal of the International Association for the Study of Obesity. 1992;16:S13-7.
[123] Nilsson AC, Östman EM, Holst JJ, Björck IM. Including indigestible carbohydrates in the evening meal of healthy subjects improves glucose tolerance, lowers inflammatory markers, and increases satiety after a subsequent standardized breakfast. The Journal of nutrition. 2008;138(4):732-9.
[124] Rault-Nania M-H, Demougeot C, Gueux E, Berthelot A, Dzimira S, Rayssiguier Y, et al. Inulin supplementation prevents high fructose diet-induced hypertension in rats. Clinical Nutrition. 2008;27(2):276-82.
[125] Parnell JA, Klancic T, Reimer RA. Oligofructose decreases serum lipopolysaccharide and plasminogen activator inhibitor-1 in adults with overweight/obesity. Obesity. 2017;25(3):510-3.
[126] Nicolucci AC, Hume MP, Martínez I, Mayengbam S, Walter J, Reimer RA. Prebiotic Reduces Body Fat and Alters Intestinal Microbiota in Children With Overweight or Obesity. Gastroenterology. 2017.

[127] Delzenne NM, Kok N, Fiordaliso M-F, Deboyser DM, Goethals FM, Roberfroid MB. Dietary fructooligosaccharides modify lipid metabolism in rats. The American Journal of Clinical Nutrition. 1993;57(5):820S-S.
[128] Kok N, Roberfroid M, Robert A, Delzenne N. Involvement of lipogenesis in the lower VLDL secretion induced by oligofructose in rats. British Journal of Nutrition. 1996;76(06):881-90.
[129] Sa'ad H, Peppelenbosch MP, Roelofsen H, Vonk RJ, Venema K. Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2010;1801(11):1175-83.
[130] Haber B, Ter Meer H-U, Hausmanns S. Cholesterol-reducing agent made of dietary fibre and cholesterol-reducing substances. Google Patents; 2003.
[131] Yamamoto Y, Takahashi Y, Kawano M, Iizuka M, Matsumoto T, Saeki S, et al. In vitro digestibility and fermentability of levan and its hypocholesterolemic effects in rats. The Journal of nutritional biochemistry. 1999;10(1):13-8.
[132] Perry RJ, Peng L, Barry NA, Cline GW, Zhang D, Cardone RL, et al. Acetate mediates a microbiome–brain–β-cell axis to promote metabolic syndrome. Nature. 2016;534(7606):213-7.
[133] Cani PD, Lecourt E, Dewulf EM, Sohet FM, Pachikian BD, Naslain D, et al. Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide production with consequences for appetite sensation and glucose response after a meal. The American journal of clinical nutrition. 2009;90(5):1236-43.
[134] Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut. 2009;58(8):1091-103.
[135] Cani PD, Knauf C, Iglesias MA, Drucker DJ, Delzenne NM, Burcelin R. Improvement of glucose tolerance and hepatic insulin sensitivity by oligofructose requires a functional glucagon-like peptide 1 receptor. Diabetes. 2006;55(5):1484-90.
[136] Delzenne NM, Cani PD. Gut microbiota and the pathogenesis of insulin resistance. Current diabetes reports. 2011;11(3):154-9.
[137] Kang SA, HONG K, Jang K-H, KIM S, LEE KH, Chang B-I, et al. Anti-obesity and hypolipidemic effects of dietary levan in high fat diet-induced obese rats. Journal of microbiology and biotechnology. 2004;14(4):796-804.
[138] Parnell JA, Reimer RA. Weight loss during oligofructose supplementation is associated with decreased ghrelin and increased peptide YY in overweight and obese adults. The American journal of clinical nutrition. 2009;89(6):1751-9.
[139] Lee SR, Hwang KT, Ji GE. The Anti-Obesity Effects of the Dietary Combination of Fermented Red Ginseng with Levan in High Fat Diet Mouse Model. Phytotherapy research. 2014;28(4):617-22.
[140] Hume MP, Nicolucci AC, Reimer RA. Prebiotic supplementation improves appetite control in children with overweight and obesity: a randomized controlled trial. The American Journal of Clinical Nutrition. 2017;105(4):790-9.
[141] Kazak H, Barbosa A, Baregzay B, da Cunha MA, Oner ET, Dekker R, et al. Biological activities of bacterial levan and three fungal β-glucans, botryosphaeran and lasiodiplodan under high glucose condition in the pancreatic β-cell line INS-1E. Adaptation biology and medicine: new developments. 2014;7:105-15.
[142] Dahech I, Belghith KS, Hamden K, Feki A, Belghith H, Mejdoub H. Oral administration of levan polysaccharide reduces the alloxan-induced oxidative stress in rats. International journal of biological macromolecules. 2011;49(5):942-7.
[143] Gao Z, Yin J, Zhang J, Ward RE, Martin RJ, Lefevre M, et al. Butyrate improves insulin sensitivity and increases energy expenditure in mice. Diabetes. 2009;58(7):1509-17.
[144] Belghith KS, Dahech I, Hamden K, Feki A, Mejdoub H, Belghith H. Hypolipidemic effect of diet supplementation with bacterial levan in cholesterol-fed rats. International journal of biological macromolecules. 2012;50(4):1070-4.
[145] Gobinath D, Madhu AN, Prashant G, Srinivasan K, Prapulla SG. Beneficial effect of xylo-oligosaccharides and fructo-oligosaccharides in streptozotocin-induced diabetic rats. British Journal of Nutrition. 2010;104(01):40-7.
[146] Chen K, Chen H, Faas MM, Haan BJ, Li J, Xiao P, et al. Specific inulin-type fructan fibers protect against autoimmune diabetes by modulating gut immunity, barrier function, and microbiota homeostasis. Molecular Nutrition & Food Research. 2017.
[147] Lightowler H, Thondre S, Holz A, Theis S. Replacement of glycaemic carbohydrates by inulin-type fructans from chicory (oligofructose, inulin) reduces the postprandial blood glucose and insulin response to foods: report of two double-blind, randomized, controlled trials. European Journal of Nutrition. 2017:1-10.
[148] Holscher HD. Dietary fiber and prebiotics and the gastrointestinal microbiota. Gut Microbes. 2017;8(2):172-84.
[149] De Preter V, Verbeke K. Functional Aspects of Prebiotics and the Impact on Human Health. 2014.

Herbal Extracts as Bioinsecticides for sustainable Agriculture

J.B. Martínez Carrión[1], **I. Córdova Guerrero**[2], **J. González Marrero**[1], **M. Macías Alonso**[1], **S. Osegueda Robles**[1] and **F. Ledezma García**[3]

[1] Instituto Politécnico Nacional, UPIIG, Av. Mineral de Valenciana, No. 200, Col. Fracc. Industrial Puerto Interior, C.P. 36275 Silao de la Victoria, Guanajuato, México
[2] Facultad de Ciencias Químicas e Ing. Universidad Autónoma de Baja California. Clz. Universidad 14418, Parque Industrial Internacional. Tijuana, B. C. CP 22390. México
[3] Agrobiotecnología del Bajío, Calle Puerta de San Diego, León, Guanajuato, México

There is no doubt that the need to improve agricultural productivity and enhance its sustainability is one of the most significant challenges facing humanity. In order to feed the dramatically growing global population, food production must increase by 70 percent. The damage caused by insect pests is one of the most important factors leading to the reduced production of major crop plant species. With this projection, combined with increasing demand for sustainable agricultural practices, research is required in order to produce more toxicologically and environmentally benign pesticides to sustain future agricultural production and global food security. Synthetic chemicals are generally used to control insect pests, which cause harmful impacts on the environment and non-target living systems including human beings. For these reasons, biopesticides are gaining increasing importance as they are alternatives to chemical pesticides and are a major component of many pest control programs worldwide. The most important biopesticides on the market in commercial terms are microbial pesticides, pyrethrum, rotenone, neem oil and various essential oils. This chapter details the benefits of biopesticides, offering a full spectrum and review of the process to identify, evaluate, and develop new biopesticides. It describes the range of oil and plant extracts that may be used in the biological control of insects, and their modes of action. Finally, the chapter describes new opportunities for developing biopesticides.

Keywords: Pest management; Pesticide; Biopesticides; Extracts; Microbial; Sustainable development

1. Introduction

Agriculture is an important resource to sustain global economical, environmental and social system. There are estimated to be around 67 000 different crop pest species (including insect, plant pathogen and weed pests) and together destroy about 30-40% of world's food production [1]. Pesticides are often the only practical way to control pests before they harm us or our crops from time immemorial. The term pesticide is defined by FAO (1990) as chemicals designed to combat the attacks of various pests and vectors on agricultural crops, domestic animals and human beings, and this broad term include terms insecticides, herbicides, fungicides, rodenticides, wood preservatives, garden chemicals and household disinfectants [2]. For centuries humans have used natural or synthetic preparations as pesticide, and their use has become more pronounced with time due to increased population paralleled with decreasing soil fertility, however their use does not always decrease crop losses. For example, despite the more than 10-fold increase in insecticide use in the United States from 1945 to 2000, total crop losses from insect damage have nearly doubled from 7 to 13% [3]. Agriculture will increasingly be expected to provide not only food for a world population continuously growing, but also crops for their conversion into renewable fuels and chemical feedstocks. For these reasons, there is no doubt that this problem impacts the worldwide economy, with the increasing pressure to improve agricultural productivity in a sustainable manner. For this reason, the global challenge is to secure high and quality yields and to make agricultural production environmentally compatible.

In the 20th century synthetic insecticides have replaced natural ones, and appeared great numbers of effective compounds as organophosphate insecticides (1960s), carbamates (1970s), pyrethroids (1980s) and herbicides and fungicides (1970s–1980s). Current insecticides act primarily on four nerve targets, which are present in animals but not plants: acetylcholinesterase (organophosphorus and methylcarbamates), voltage-gated sodium channels across the nerve membrane (pyrethoids and DDT), acetylcholine receptor (neonicotinoids), γ-aminobutyric acid receptor and glutamate-receptor chloride channels (polychlorocycloalkanes, avermectins, and spinosyns) [4-6]. In the case of bioinsecticides, their mechanisms of action are extremely variable and it is not always understood [7].

However, the intensive and indiscriminate use of pesticides has resulted in some detrimental consequences on environment such as groundwater pollution, river eutrophication, toxicity to non-target organisms, and the selection of strains not only resistant to the selecting compounds but also cross-resistant to other pesticides acting at the same target. Many of the pesticides currently being used have the tendency to survive in plants for a long time. They also enter the food chain and are found in meat and dairy products and remain as residue in the soil and ecosystem for long periods of time [8-9]. It also has negative impact on health with human poisonings and their related illnesses [10]. Keeping all these facts in mind, together with the new regulatory requirements and the demand of the public for safer foods, should stimulate the development of eco-friendly alternatives to chemical pesticides to generate higher quality and greater

quantity of agricultural products in a sustainable mode. These new products must be effective, biodegradable, environmentally safe and innocuous.

1.1 Biopesticides

In 2003 the Advisory Committee on Pesticides (ACP) investigated the prospects for developing alternatives to pesticides in the UK. The report recognised that while there are a number of difficulties in developing alternatives to pesticides, many of these alternative methods are viewed favourably by large sections of the general public, and therefore this presents a strong argument for their development and wide usage. However, from the farmers' and retailers' point of view their benefits are less certain. Their efficacy is often lower than conventional pesticides, and they are more variable in effect than conventional pesticide sprays (Advisory Committee on Pesticides, 2003) [11].

In this context, biopesticides are a particular group of crop protection tools used in an integrated pest management. In very general terms, according to the United States Environmental Protection Agency, it could be defined as living organisms and/or their natural products that prevent, control or suppress pest populations by nontoxic mechanisms, which pose less threat to the environment and to human health.

Some common benefits and disadvantages of biopesticides in comparison with conventional pesticides are shown in the table below (Table 1) [12]. In sum, biopesticides tend to be less toxic, more quickly biodegradable, and more targeted to the specific pest. Biopesticides are often designed to control a pest population to a manageable level rather than completely eradicate a target pest. These technical differences translate into benefits to humans and ecosystems including increased food safety, worker safety, and reduced concerns for development of pest resistance to existing control tools.

Table 1 Pros and cons of biopesticide active ingredients in comparison with conventional pesticides.

Benefits	Disadvantages
Less toxic and less environmental load Reduces their impact on beneficial and non target organisms.	*Toxicity* Several botanical insecticides are more toxic to humans and fish than a number of synthetically derived insecticides.
Rapid biodegradation Rapid degradation under environmental conditions such as sunlight, humidity, and rainfall.	*Limited field persistence and a short shelf life* More frequent applications may be necessary. Have relatively critical application times.
More targeted to specific pest Generally nontoxic to humans, mammals, and bees.	*Narrower target range*
Specific mode of action Generally affect only the target pest and closely related organisms, in contrast to broad spectrum pesticides.	*Specific mode of action* Care to choose the product that targets the pest you need to control.
Manage rather than eradicate Maintain ecological balance. Suppress, rather than eliminate, a pest population.	*Slower acting* The time from exposure to morbidity and death of the target insect may be 2 to 10 days.
Often effective in very small quantities and often decompose quickly Thereby resulting in lower exposures problems.	*Costs and availability* Generally more expensive than synthetically derived insecticides. Lack of sales and problems associated with providing consistent product.
Minimal impacts on plants Most botanical insecticides are not harmful to plants when applied according to the label directions.	*Lack of efficacy data* Insufficient data exist on botanical insecticides, both in terms of effectiveness and chronic toxicity.

A general classification of biopesticides is based in three different categories according to the type of active ingredient used: (i) plant-incorporated protectants, (ii) microbial pesticides, and (iii) biochemical pesticides [13]. In general, there are significant differences in the mode of action between microbial and biochemical (Table 2) [14]. The majority of the biopesticide market, approximately 90%, are living organisms. These include biofungicides (*Trichoderma*), bioherbicides (*Phytophthora*) and bioinsecticides (*Bacillus thuringiensis*).

A more recently introduced term is biocontrol agents instead of biopesticide, which are classified into four groups: (i) macrobials, (ii) microbials, (iii) natural products, and (iv) semiochemicals (insect behavior-modifying agents). Among all the biocontrol agents, the most important products are microbials (41%), followed by macrobials (33%), and finally other natural products (26%) [15].

In addition to being categorized by the active ingredient, biopesticides can be categorized by the target pest, such as insecticides to manage insect populations and fungicides to manage fungus.

The need for more environmentally-friendly forms of pesticide is therefore greater than ever. Therefore, biopesticides have gained increasing importance due to their potential in developing environmentally friendly and safe approaches and tactics for pest management, in particular for the development of products that could replace synthetic chemical pesticides [16].

Table 2 Categories of biopesticides [19].

Types	Actives Ingredients	Mode of action	Examples [13]
Plant-incorporated protectants	Genetic material added to the plant, which is commonly known as a transgenic crop or a genetically modified organism.	Plants themselves can produce the proteins and protect themselves from insects without any external pesticide.	Coat protein gene of *Plum Pox* virus *Bacillus thuringiensis Vip3Aa20, Cry1A.105* and Cry2Ab2
Microbial pesticides	Naturally occurring or genetically controlled microorganism. The active ingredient can be either the spores or the organism itself.	Relatively specific for its target pests. Act by exploitation, competition, antibiosis, lysis and/or induced resistance.	*Ulocladium oudemansii* *Bacillus thuringiensis* *Beauveria bassiana* *Trichoderma asperellum* *Trichoderma gamsii*
Biochemical pesticides	Chemicals either extracted from natural sources or synthesized to have the same structure and function as the naturally occurring chemicals. They control pests by non-toxic mechanisms.	Act by contact, ingestion, systemic action, suffocation and/or attraction/repulsion. Include substances that interfere with growth or mating, such as plant growth regulators, or substances that repel or attract pests, such as pheromones.	They include substances, such as insect sex pheromones, as well as various scented plant extracts, or fatty acids. Cold pressed *Neem Oil* Extract of *Chenopodium ambrosioides* Saponins of *Quillaja saponaria*

The term "natural" conveys a sense of wholesomeness or safety, however it is important to indicate that based on the LD_{50}, a number of registered botanicals are toxic to fish, beneficial insects and mites, and mammals. In fact, several botanical insecticides have a lower LD_{50} than the synthetically derived insecticides carbaryl (Sevin) and malathion (Table 3). Although naturally occurring insect toxins are extracted from plants, "natural" does not necessarily imply "safe" or "nontoxic." For example, arsenic, strychnine, lead, mercury, nicotine, and other similar compounds used historically as pesticides technically qualify as natural. Today no one considers these compounds wholesome or safe. In most cases, botanical insecticides are less toxic to humans than synthetically derived insectides [17]. For example, *Nicotiana tabacum* is the most toxic of the botanical insecticides, with an LD_{50} between 50 and 60 mg/kg. It is extremely harmful to humans. Nicotine, a fast-acting nerve toxin, works as a contact poison.

Biopesticides offer an environmentally sustainable approach to increase crop production and health. There is a tremendous amount of work and research occurring in this field. The topic of genetically modified organisms is a broad topic that warrants its own investigation, and will not be covered in this report. In this chapter, we intend to present an overview of the most significant advances described in the latest literature concerning the challenges and opportunities for the development of biopesticides. We mainly focus on Biochemical pesticides, including the progress achieved in the discovery process of new potential biopesticides. For the purposes of this report, biopesticides have been categorized in a manner similar to that used by the EPA.

Table 3 Ranking of pesticide with each other and commonly used synthetically derived insecticides, based on their toxicity rating (oral LD_{50}).*

Common Name	Active compounds	Acute Oral LD_{50}	Acute Dermal LD_{50}	Bees	Type of pesticide
Allium sativum	Allicin	3034	–	Non-toxic	Insecticide
Bacillus thuringiensis	–	>5,000	>2,000	Non-toxic	Insecticide
Capsicum annum	Capsaicin	148-161	>512	Toxic	Insecticide
Citronella oil	Citronellal, geraniol	7200	4700	Low-toxic	Insecticide, Herbicide
Citrus oil	Limonene, linalool	4,000-5,000		Low-toxic	Insecticide
Clove essential oil	Eugenol	2650	–	Low-toxic	Insecticide, Herbicide
Kinoprene	–	4,950	9,000	Non-toxic	Insecticide
Neem oil	Azadirachtin, dihydroazadirachtin	4,200	2,000	Low-toxic	Insecticide, Acaricide, Fungicide

Nicotiana tabacum	Nicotine	55	–	High-toxic	Insecticide
Pyrethrin	Esters of chrysanthemic and pyrethric acid	1,500	>1,800	High-toxic	Insecticide, Acaricide
Rotenone	Rotenone, deguelin	350	940	High-toxic	Insecticide, Acaricide
Ryania speciosa	Ryanodine, 9,21-dehydro ryanodine	750	–	Low-toxic	Insecticide
Sabadilla	Cevadine, veratridine	5,000	–	High-toxic	Insecticide
Warfarin	–	3	–	Non-toxic	Rodenticide
DDT	–	113	2,510	High-toxic	Insecticide
Malathion	–	2,800	4,100	High-toxic	Insecticide
Carbaryl	–	246-283	4,000	High-toxic	Insecticide

* LD_{50}: It is the amount of a material, given all at once, which causes the death of 50% of a group of test animals. LD_{50}values are expressed as milligrams per kilogram (mg/kg), which means milligrams of chemical per kilogram of body weight of the animal.

2. Living organisms as Insecticides

Microbial products may consist of the organisms themselves and/or the metabolites they produce to control of pest insects, plant pathogens and weeds. Over 400 species of fungi and more than 90 species of bacteria which infect insects have been described. These microorganisms, are legally considered biopesticides and are regulated as such [13]. In the case of although multicellular organisms such as nematodes, they are not regulated as pesticides. The advantage to using biological products is their higher selectivity, therefore they are lower or no toxic to non pathogenic organism, to wildlife, humans, and other organisms not closely related to the target pest, in comparison to conventional chemical pesticides. A limitation of several types of microbial insecticides is that heat, desiccation or exposure to ultraviolet radiation reduces their effectiveness, and consequently, proper timing and application procedures are especially important for some products [18]. Bacteria, fungi, oomycetes, viruses and protozoa are all being used for the biological control. In Table 4 we described some special characteristics of these types of microbial insecticides.

Table 4 Types of microbial insecticides [19-21].

Bacteria	
Uses	Bacterial biopesticides are the most common and cheaper form of microbial pesticides. They are effective against a wide spectrum of plant diseases, nematodes, insects, and weeds.
Mode of action	Must be eaten to be effective. Bacteria disrupt the digestive system by producing endotoxins that are often specific to the particular insect pest.
Examples	*Bacillus sphaericus* used to control certain mosquito species. *Bacillus subtilis* and *Bacillus pumilus* increase yield and prevent plant diseases by outcompeting plant pathogens in the rhizosphere, producing anti-fungal compounds.
Fungi	
Uses	Useful to control plant diseases caused by other fungi, bacteria or nematodes, as well as some insect pests and weeds. They may be applied in the form of conidia or mycelium which sporulates after application. Very useful for insecticide resistant management.
Mode of action	The mode of action is varied and depends on both the pesticidal fungus and the target pest. One advantage of fungal biopesticides is that they do not need to be eaten to be effective.
Examples	*Trichoderma spp.* colonize plant roots, without harming the plant. It can out-compete pathogenic fungi for food and space, and in the process can stimulate plant host defenses and affect root growth. *Beauveria bassiana* is effective in controlling troublesome crop pests such as aphids, thrips and whitefly – even chemical pesticide-resistant strains such as Q-Biotype Whitefly. Have been reported the use of *Metarhizium anisopliae* against adult *Aedes aegypti* and *Aedes albopictus* mosquitoes.
Protozoa	
Uses	Protozoan pathogens, single-celled eukaryotic organisms, naturally infect a wide range of insect hosts.

Mode of action	Must be eaten to infect an insect. They enter the insect via the gut wall, spreads to various tissues and organs, and they multiply, sometimes causing tissue breakdown and septicemia. One important consequence is a reduction in the number of offspring produced by infected insects.
Examples	*Nosema locustae* infects at least 90 species without affects to humans and other mammals, as well as the over 250 natural predators of grasshoppers. *Nosema pyrausta* infects several insect species, including European corn borer, for which it can be an important natural control. *Vairimorpha necatrix* has a wide host range among caterpillar pests, including corn earworm and European corn borer, various armyworms, fall webworm, and cabbage looper.
	Viruses (baculoviruses)
Uses	Baculovirus is the main virus that is commercially used. It is a special family of naturally-occurring viruses, to which about 100 insects and some related arthropods are susceptible. They are very specific. Baculoviruses used consist of DNA surrounded by a protein coat (nucleocapsid), which is itself embedded in a protein "microcapsule" or occlusion body (OB) that provides some protection from degradation in the environment.
Mode of action	Upon ingestion by a susceptible caterpillar, OBs are dissolved within the alkaline midgut, releasing nucleocapsids that infect the cells lining the midgut. The viral DNA replicates in the nuclei of the host cells and then spreads throughout the body of the larvae. The infected insect stops feeding within a few days, dies and disintegrates.
Examples	The granulovirus of the codling moth *Cydia pomonella*, it is the active ingredient of about a half-dozen products sold worldwide, and limits codling moth populations and damage in pome fruits while preserving beneficial insects and minimizing chemical residues. Preparations of *Heliothis zea* nucleopolyhedrovirus infects many species of *Helicoverpa* and *Heliothis* genera. These products provided control of not only cotton bollworm, but also of pests belonging to these genera attacking soybean, sorghum, maize, tomato and beans. *Anticarsia gemmatalis* nucleopolyhedrovirus control the velvetbeen caterpillar in soybean.
	Yeast
Uses	*Cryptococcus* and *Candida* species have been investigated for their usefulness in controlling plant diseases.
Mode of action	Works primarily through competition for nutrients and pre-colonization of plant wound sites. It exist evidence that it produces enzymes that can degrade fungal cell walls and stimulate plant host defence pathways in freshly harvested fruit.
Examples	*Candida oleophila* (strain O) is an effective biopesticide for the control of post-harvest fruit rots. It is applied to apples and pears after harvest — but before storage — to control particular fungal pathogens. The yeast serves as an antagonist to fungal pathogens such as gray mold (*Botrytis cinerea*) and blue mold (*Penicillium expansum*), which cause post-harvest decay.

We refer to data published annually by the State of California's Department of Pesticide Regulation to discuss the extent to which each of the more important botanical insecticides are used [22]. At present *Bacillus thuringiensis* is the most studied entomopathogenic species and some of its crystal producing strains have certainly represented the main active substances used for the microbial pest management during the last decades. Additional microorganisms as *Beauveria bassiana* have limited use in various countries (table 5).

Table 5 Microbial treatment most often used against insect pests.

	Bacillus thuringiensis (Bt) [1, 23-24]
Active compounds	Bt is a ubiquitous Gram-positive, rod-shaped and sporulating bacterium that synthesize crystal (Cry) and cytolytic (Cyt) toxins, (also known as δ-endotoxins), at the onset of sporulation and during the stationary growth phase as parasporal crystalline inclusions.
Mode of action	Once ingested by insects, these crystals are solubilized in the midgut, the toxins are then proteolytically activated by midgut proteases and bind to specific receptors located in the insect cell membrane leading to cell disruption and insect death. The δ-endotoxins are host specific and can cause host death within 48 h.
Uses	The most world-wide used biopesticide. Microbial Bt biopesticides consist of bacterial spores and δ-endotoxins crystals mass-produced in fermentation tanks and formulated as a sprayable product. It feeds on the larval stages of insect pests such as mosquitoes, Colorado potato beetles, and cabbage loopers. It has been used for pest management on fruit and vegetable crops such as maize, soya bean and cotton.
Advantages	It does not harm vertebrates and is safe to people, beneficial organisms and the environment. High level of selectivity, so different strains of Bt are effective against specific pests. For example, *Bt. var. kurstaki* kills caterpillars, while *Bt. var. israelensis* is for mosquitoes and other fly larvae. Useful where resistance to synthetic chemical insecticides is a problem.
Disadvantages	*B. thuringiensis* products can be sensitive to sunlight, giving them a very short period of effectiveness, so multiple applications are often needed for adequate management of the pest. Bt is only effective when ingested.
Total pesticide used in California (2014)	*Bt (berliner), subsp. kurstaki*, strain SA-11: 80,311 lb (36,428 Kg) *Bt, subsp. aizawai*, strain abts-1857: 48,784 lb (22,128 Kg) *Bt, subsp. israelensis*, strain am 65-52: 42,746lb (19,389 Kg) *Bt*, *subsp. kurstaki*, strain abts-351: 111,273lb (50,472 Kg)

Beauveria bassiana[1, 25-26]	
Active compounds	The key secondary metabolites produced by *Beauveria bassiana*, a soilborne fungus, are beauvericin, bassianolide, bassianin, tenellin and cyclosporin A.
Mode of action	*B. bassiana* can be applied as a spore. Once the spores have contact with the insect exoskeleton, they grow hyphae (long, branching vegetative appendages) that secrete enzymes, which in turn dissolve the cuticle (outermost layer of the skeleton). These fungal hyphae then grow into the insect, feed on its body tissue, produce toxins, and reproduce. It takes up to seven days for the insect to die.
Uses	Effectively to control thrips, aphids, whitefly, caterpillars, beetles, and subterranean insects like ants, spider mites and termites.
Advantages	It does not harm vertebrates and is safe to people, beneficial organisms and the environment. The strain used and sold commercially does not affect honey bees
Disadvantages	*B. bassiana* can be sensitive to sunlight, giving them a very short period of effectiveness. Its spores will infect many non target beneficial insects too. Toxic metabolitesof *B. bassiana* may enter the plants. In minor cases conidia of *B. bassiana* have allergenic potential.
Total pesticide used in California (2014)	*Beauveria bassiana* strain GHA: 2,746lb (1,246 Kg)

3. Plant Extracts as Natural Insecticides

In the search for alternative solutions to crop protection problems, the interest in plants extracts has increased. Throughout evolution plants have developed an effective defence system against microbial attack or insect/animal predation, including the production of low molecular mass secondary metabolites with antimicrobial activity, which are synthesized *de novo* after stress and are collectively known as phytoalexins, which have toxic, repellent, and/or antinutritional effects on pest [27-28]. This plethora of chemical-defensive compounds include alkaloids, phenolics and terpenoids, and could be used to combat insect pests and disease pathogens. Their mechanisms of action can vary, especially when the activity is due to a complex mixture of compounds that can be toxic or repellent to the target organisms and cause developmental changes including sterility, reduced growth, and altered behavior. Till date, over 2000 plant species have been known to produce secondary metabolites of value in biological pest control programs, and many of these plants are used by farmers in developing countries. Only a small percentage of plants have been screened for pesticidal activity, thus potentially useful biological compounds remain undiscovered [29-30].

Of particular interest are essential oils, which are generally composed of complex mixtures of mono and sesquiterpenes. In the nature those compounds plays an important role in the protection of the plants against bacteria, fungi, virus, insects and others herbivores. For these reasons, certain essential oils are considered to be an alternative means of controlling many harmful insects, especially against small, soft-bodied insects and mites that are immobile or slow-moving (e.g., aphids, scales, leafhopper nymphs, whiteflies). Essential oil products are generally considered as broad spectrum because of the presence of several active ingredients that operate through several modes of action, including larvicidal and antifeeding, inhibit molting and respiration, reduce growth and fecundity, and display phototoxicity [31-33].

Humans have made use of botanical insecticides to control pests for hundreds of years. These products can be marketed in one of three ways: (1) preparations of the crude plant material, ground into a dust or powder; (2) extracts from plant resins, formulated into liquid concentrations; and (3) isolation of the pure chemicals obtained from plants by extraction or distillation.

We refer to data published annually by the State of California's Department of Pesticide Regulation to discuss the extent to which each of the more important botanical insecticides are used [22]. In the present there are four major types of botanical products used for insect control (pyrethrum, rotenone, neem, and essential oils), along with three others in limited use (ryania, nicotine, and sabadilla). Additional plant extracts and oils (e.g., garlic oil, *Capsicum* oleoresin) see limited (low volume) regional use in various countries (table 6) [34].

Table 6 Alternative plant extracts options for control of insect pests.

Neem-based insecticides [35-36]	
Active compounds	Limonoids, mainly azadirachtin A and B, obtained from different parts of the plant (mainly the seed) of the neem tree (*Azadirachta indica* A. Juss). The best solvents for the extraction of azadirachtin A are water and methanol.
Mode of action	Antifeedancy, fecundity suppression, ovicidal and larvicidal activity, growth regulation and repellence, also at low dosages. Azadirachtin reduces the level of the insect hormone Ecdysone, which affect corpus cardiacum and block reproductive and growth processes in most insects causing sterility in females and degenerative changes in male testis due to disturbance in insect metabolism.

Uses	For more than 400 insect pests of medical, veterinary and agricultural importance including pests species belonging to Lepidoptera, Diptera, Coleoptera, Homoptera and Hemiptera. Range of crops.
Advantages	Non-toxic to humans and animals, including useful insects like bees, exhibits fewer chances of resistance, due to its multiple mode of action on insects, and have no residual effect on agricultural products. In most tests neem products performed equally or sometimes better than synthetics. Neem based pesticides are easy to prepare, cheap and highly effective.
Disadvantages	Limited outdoor crop studies.
Total pesticide used in California (2014)	Clarified hydrophobic extract of neem oil: 196,906 Pounds (89315 Kg) Margosa Oil (Obtained from the fruits and seeds of neem: 22,547 Pounds (10227 Kg) Azadirachtin: 3,999 Pounds (1814 Kg)
Garlic essential oil [37-38]	
Active compounds	The principal biologically active compound produced by garlic (*Allium sativum*) is allicin, a sulphur containing (thiosulphonate)
Mode of action	Some studies have proved that garlic oil has fungicidal, repellent, insecticidal, nematicidal, and antibiotic properties.
Use	It is used on a wide range of pests, including aphids, thrips, leafhoppers and caterpillars. Crops: avocados, citrus, ornamentals.
Advantages	Garlic exhibits antibacterial, antifungal, amoebicidal and insecticidal qualities. Garlic is not persistent in the environment.
Disadvantages	Toxic to bees and fish. Degradation rapid due to sunlight and ultraviolet light. Thus, it is not recommend as an all-purpose spray for outdoor use. Limited studies.
Total pesticide used in California (2014)	Garlic essential oil: 1,392 Pounds (631 Kg)
Chenopodium ambrosioides near ambrosioides[39-40]	
Active compounds	Terpenes: α-terpinene, d-limonene and p-cymene
Mode of action	The active ingredient is lipophilic; attracted to the oily outer surfaces of target pests, and works to kill target pests in three ways: 1. Collapses trachea causing asphyxiation; 2. Destroys cuticle layer causing desiccation; 3. Anti-feeding properties.
Use	Controls soft-bodied sucking pests including thrips, whiteflies, aphids, mites, leaf hoppers, leafminers, and psyllid, in high-value fruits and vegetables. It also deters feeding and reduces the spread of viruses.
Advantages	Resistance development reduced/unlikely due to three different modes of action. Active against all lifecycle stages – eggs to adults. Safe for workers, the environment, and neighbours. Field trials confirm safety on Bees (*Apis mellifera*).
Disadvantages	Good coverage is essential. Nil/Limited outdoor field studies.
Total pesticide used in California (2014)	*Chenopodium ambrosioides* near ambrosioides essential oil: 17,504 Pounds (7940 Kg)
Canola oil [41]	
Active compounds	Refined vegetable oil obtained from the seeds of four species of rape plants of the family *Cruciferae* (mustard family): *Brassicanapus*, *B. juncea*, *B. rapa* and *B. campestris*.
Mode of action	Repels insects by altering the outer layer of the leaf surface or by acting as an irritant.
Use	Citrus, corn, fruit trees, nut trees, sugar beets, soybeans, tomatoes, vegetables, figs, melon, olives, small fruits, alfalfa, bedding plants, ornamentals, and houseplants.
Advantages	The oil is biodegradable and leaves little residue and does not taint the crops.
Disadvantages	Application when temperatures are high (above 30–35°C) and/or humidity is low may cause leaf scorch and interfere with plant respiration. Reapplication may be necessary depending on weather conditions. Limited outdoor crop studies.
Total pesticide used in California (2014)	Canola oil: 34 Pounds (16 Kg)

Oriental Mustard Seed Allyl isothiocyanate [42-43]	
Active compounds	Seeds of brown or oriental mustard (*Brassica juncea*),with high concentrations of sinigrin (ally lglucosinolate, *S*-glucopyranosyl thiohydroximates).
Mode of action	The glucosinolates present in the *Brassica* species are hydrolyzed, upon contact with water, by thioglucosidases called myrosinases to isothiocyanates, which have been proven toxic against insects, fungi and nematodes.
Use	Wide spectrum of anti-microbial effects and repellents against certain species of insect. Insecticidal. Nematicidal activity on *Heterodera glycines*, *Pratylenchus neglectus*, *Heterodera schachtii*, *Pratylenchus penetrans*, *Meloidogyne incognita*, *Meloidogyne hapla* and *Caenorhabditis elegans* in the laboratory; and on *Pratylenchus penetrans* on sweet corn in a greenhouse.
Advantages	The oil is biodegradable and leaves little residue and does not taint the crops.
Disadvantages	The effectiveness of these practices in suppressing nematode populations in soil has not been conclusive.
Total pesticide used in California (2014)	-
Pyrethrum [44-45]	
Active compounds	Oleoresin extracted from the dried flowers of *Tanacetum cinerariaefolium*. Two pyrethrins are most prominent, pyrethrin-I and pyrethrin-II. The pyrethrins have another four different active ingredients, Cinerin I and II and Jasmolin I and II.
Mode of action	They are contact poisons which induce a quickly neurotoxic effect in insects. They delay the closure of voltage-gated sodium ion channels in the nerve cells of insects, resulting in repeated and extended nerve firings. This hyperexcitation causes the death of the insect due to loss of motor coordination and paralysis. The mechanism is qualitatively similar to that of DDT and many synthetic organochlorine insecticides.
Use	Broad spectrum of insect pests of medical, veterinary and agricultural importance.
Advantages	They are one of least toxic domestic insecticides available (rat oral acute LD_{50}value~1500 mg kg^{-1}). Broad spectrum of insecticides.
Disadvantages	Pyrethrin is extremely toxic to aquatic life. Natural pyrethrins are highly fat soluble, but are easily degraded and thus do not accumulate in the body. These compounds are toxic, and affect their feeding behavior. Pyrethrins are degraded by the combination of sunlight and air, and therefore present low persistence.
Total pesticide used in California (2014)	Pyrethrum accounted for 80% of all botanicals used that year, but only 27% of that amount was used in agriculture.
Heliopsis longipes [46-47]	
Active compounds	Alkamides as *N*-isobutil-2*E*,6*Z*,8*E*-decatrienamide and *N*-isobutil-2*E*,6*Z*,8*E*-decatrienamide
Mode of action	Similarly to pyrethrins, it is a potent voltage-dependent blockers of sodium channel, with paralyzing effects.
Use	Control populations of insect vector transmitters of several diseases that affect the human health as *Anopheles albimanus* (LC_{50} 4.24mg/L) and *Aedes aegypti (*LC_{50} 7.38 mg/L).
Advantages	Non-toxic to humans and animals, including useful insects like bees.
Disadvantages	It is too unstable to be used as products directly. Limited outdoor crop studies.
Total pesticide used in California (2014)	-

4. Process to identify, evaluate, and develop new biopesticides from natural products

Development of new and useful biopesticides has continued to increase rapidly since the mid-1990s. Cantrell *et al*. [48] found that from the years 1997−2010, 277 new active ingredients (NAI) were registered as conventional pesticide or biopesticide for the United States Environmental Protection Agency (EPA). When examining conventional pesticides and biopesticides combined, and considering that natural products, synthetic natural derived, and biological all have origins from natural product research, it can be argued that their combined impact with the EPA from 1997 to 2010 accounted for 69.3% of all NAI registrations. It shows clearly that natural products play an important role in discovery and development of new products. However, nature's diversity has not been efficiently explored for discovery of new natural-product pesticides.

Different approaches to discovery and development of pesticide compounds using higher plants can be distinguished: a. random selection followed by chemical screening; b. random selection followed by one or more biological assays; c. follow-up of biological activity reports; d. follow-up of ethnobotanical information [49]. Once the interest

lignocellulosic materials is chosen, new active substances should be isolated and identificated. Bioassay-guided fractionation has proven successful as a well-established platform to isolate and characterize active constituents present in natural product extracts; however, sometimes such an approach requires multiple chromatographic steps and large amounts of biological material. It is necessary to develop new technologies to accelerate this phase. Process such as automated separation techniques, high-throughput screening and combinatorial chemistry are revolutionizing lead compounds discovery. Metabolomics offers a new way of studying complex molecular problems and is particularly applicable for natural products research. [50].

The most common factors affecting extraction processes are matrix properties of the plant part, solvent, temperature, pressure and time. Traditional techniques such as solid-liquid or Soxhlet extractions have been used for many decades, but they are time-consuming and require relatively large quantities of solvents. Also, due to the common extractive steps used by these techniques, including heating, boiling, or refluxing, a loss of metabolites due to ionization, hydrolysis, and oxidation occurs during the procedure [51]. In recent years, some new more selective and environmentally friendly techniques were successfully proposed for extraction of metabolites. Non-conventional methods, which are more environmental friendly due to decreased use of synthetic and organic chemicals, reduced operational time, and better yield and quality of extract. They include ultrasound, pulsed electric field, enzyme digestion, extrusion, microwave heating, ohmic heating, supercritical fluids extraction, and accelerated solvents have been studied [52].

After the active ingredients with crop protection properties have been purified, and its structure have been determined by spectroscopic techniques, their biological activity must be supported by numerous bioassays both *in vitro* and *in vivo*. *In vivo* screens give an early realistic read-out of efficacy in the practical context and *in vitro* tests have particular utility in unearthing new mode of action targets. Criteria that distinguish good bioassays are: reproducibility; linearity over a reasonable dose or concentration range; and predetermined endpoints. However, after the new compound has demonstrated in vitro activity against the insect, other numerous criteria must be satisfied. It is essential to determinate the chemical, toxicological, mode of action and environmental properties of new compounds in the development process, and before trying to commercialize them [53].

The final step is scale-up of the production. Botanical extracts are well-recognized sources of active molecules. However, botanical extracts are not always well accepted because of issues concerning active compound standardization, and quality control. Standardization of herbal medicines is the process of prescribing a set of standards or inherent characteristics, constant parameters, definitive qualitative and quantitative values that carry an assurance of quality, efficacy, safety and reproducibility. It is of great interest to develop well-characterized extracts in order to achieve biochemical and functional consistency between batches [54]. However, several problems not applicable to synthetic drugs often influence the quality of herbal drugs, for instance: Herbal drugs are usually mixtures of many constituents; the active principle(s) is (are), in most cases, unknown; selective analytical methods or reference compounds may not be available commercially; plant materials are chemically and naturally variable; chemo-varieties and chemo cultivars exist; the source and quality of the raw material are variable. The deployment of modern analytical tools in testing the various quality parameters for an effective quality control herbal product cannot be over emphasized. The assurance of the safety and efficacy of an herbal drug requires monitoring of the quality of the product from collection through processing to the finished packaged product [55].

Finally, it is important to note that the formulation of the pesticide determine the overall efficacy, increase product stability and viability of the chosen substance once applied in field conditions. In order to choose the correct formulation one must take into account several factors such as physicochemical and biological properties, mode of application, the crop to be treated and economic considerations. Several commonly used biopesticide formulations include dry and wet powder, granules and pellets. Granules can protect the active agent from desiccation and also provide basic food for the agent, however powder is easy to apply by suspending it in water and also can cover a wide area of application.

At the present it is fundamental the development of new formulations to improve the potency, stability, and the safety of these systems to humans and the environment. The use of nanotechnology and controlled-release formulations could offer a way of developing new formulations with smaller quantities of active compound to be used more effectively with a minimization of environmental damage [56-57].

5. Conclusions and new opportunities for developing biopesticides

Today, biopesticides are an emerging technology. Worldwide there are about 1400 biopesticide products being sold, which are based on 68 active substances registered in the EU and 202 in the USA, with global market of approximately $3 billion in value, with a compound annual growth rate (CAGR) of 8.64% (compared with 3% for synthetic pesticides) which is expected to produce a global market of $10 billion by 2017. It is clear that the development of the biopesticides market depend on economic terms: a. Company producing these products should have profits; b. The comparative efficacy and the cost:benefit ratio of natural and synthetic insecticides should be favorable to the first one [1, 58]. We show in the table 7 various features which could be fundamental for the development of this technology.

Table 7 Features which could be fundamental for the development of this technology.

Advantages
Lower development price [58]. The costs associated with the development of a novel synthetic pesticides typically requires $250 million and nine years of research and regulatory approval, while a biopesticide needs less than $10 million and four years for the same process.
Emerging resistance in insects to conventional agrochemical insecticides [59]. Resistances to pesticides currently available are wide spread, while it takes longer for insects to develop a resistance to biopesticides because they have new and multiple modes of action.
Disadvantages
Regulatory barriers to biopesticide commercialization [1]. It is very expensive to prepare the biopesticide registration data portfolio, which includes information about mode of action, toxicological and ecotoxicological assays or host range testing, among others.
High competition with synthetic pesticides. It is fundamental to carry out *in vitro*, *in vivo* and field tests, to demonstrate the effectiveness of the product.
Highly specific activity. These products have a smaller market than those for products with broad spectrum activity, as chemical substance or some biopesticides (e.g. *Bacillus thuringiensis*, azadirachtins, etc.). This problem often forces biopesticide use in conjunction with conventional agrochemicals.

There is an increasing global search for environment friendly agrochemicals. However, the biopesticide production must overcome important problems which include slower pest control and higher manufacturing costs compared with conventional agrochemicals, as well as regulatory problems for their commercialization. Production technology of biopesticides should be improved, along with the implementation of quality systems for the standardization and uniformity of the process. The commercial biopesticide must be reliable, specific, indigenous, and replicable in its activity. Moreover, extensive research should be conducted in the formulation of the product to prevent degradation in the environment and to extend the shelf life of the biopesticide. Topics of current scientific interest in formulation involves lyophilized preparations of biopesticides, or the development of oil-in-water microemulsions as a nano-pesticide delivery system, in order to improve the field persistence, reduce the use of organic solvent and increase the dispersity, wettability and penetration properties of the droplets. Developed formulation should allow for long storage periods, and it should be compatible with crop production practices and equipment.
In conclusion, if the industry can obtain high quality products with the ability to act for long time in field conditions, the bioinsecticides will be able to compete with the agrochemical insecticides currently available and gradually overtake the market.

Acknowledgements The support by National Council on Science and Technology of Mexico (CONACYT), Secretary of Innovation, Science and Higher Education of Guanajuato (SICES) and Research and Postgraduate Secretary of the National Polytechnic Institute (SIP-IPN) is gratefully acknowledged.

References

[1] Chandler D, Bailey AS, Tatchell GM, Davidson G, Greaves J, Grant WP. The development, regulation and use of biopesticides for integrated pest management. Philosophical Transactions Royal Society B: Biological Sciences. 2011; 366:1987-1998.
[2] Bailey KL, Boyetchko SM, Längle T. Social and economic drivers shaping the future of biological control: a Canadian perspective on the factors affecting the development and use of microbial biopesticides. Biological Control. 2010; 52:221-229.
[3] Pimentel D. Environmental and economic costs of the application of pesticides primarily in the United States.Environment, development & sustainability.2005; 7:229–252.
[4] Rajashekar Y, Shivanandappa T. Mode of action of the natural insecticide, decaleside involves sodium pump inhibition. PLoS ONE. 2017; 12:e0170836.
[5] Casida JE. Pest toxicology: the primary mechanisms of pesticide action. Chemical Research in Toxicology.2009; 22:609-619.
[6] Casida JE, Durkin KA. Pesticide chemical research in toxicology: Lessons from nature. Chemical Research in Toxicology. 2017; 30:94−104.
[7] Aliferis KA, Jabaji S. Metabolomics - A robust bioanalytical approach for the discovery of the modes-of-action of pesticides: A review. Pesticide Biochemistry & Physiology.2011; 100:105-117.
[8] Lichtfouse E, Navarrete M, Debaeke P, Souchere V, Alberola C, Menassieu J. Agronomy for sustainable agriculture. A review. Agronomy for Sustainable Development.2009; 29:1–6.
[9] Gerhardson B. Biological substitutes for pesticides. Trends in Biotechnology.2002; 20:338–343.
[10] Paoletti MG, Pimentel D. Environmental risks of pesticides versus genetic engineering for agricultural pest control. Journal Agricultural & Environmental Ethics.2000; 12:279–303.
[11] Final Report of the Sub-group of the Advisory Committee on Pesticides on Alternatives to Conventional Pest Control Techniques in the UK: a Scoping Study of the Potential for Their Wider Use. York, UK: ACP;2003.
[12] http://advancinggreenchemistry.org/wp-content/uploads/Green-Chem-and-Sus.-Ag.-the-Role-of-Biopesticides.pdf
[13] What are biopesticides? http://www.epa.gov/pesticides/biopesticides/whatarebiopesticides.htm

[14] Villaverde JJ, Sevilla-Morán B, Sandín-España P, López-Goti C, Alonso-Prados JL. Biopesticides in the framework of the European Pesticide Regulation (EC) No. 1107/2009. Pest Management Science.2014; 70: 2–5.
[15] Guillon ML. Regulation of biological control agents in Europe. In: Roettger U, Reinhold M, editors. International symposium on biopesticides for developing countries; 2003 Oct 28-30; Turrialba, Costa Rica.Turrialba: CATIE; 2004.p. 143–147.
[16] Copping LG, Menn JJ. Biopesticides: a review of their action, applications and efficacy. Pest Management Science.2000; 56:651–676.
[17] Wiesbrook ML. Natural indeed: Are natural insecticides safer and better than conventional insecticides? Illinois Pesticide Review.2004; 17: 1–8.
[18] Lacey LA, Frutos R, Kaya HK, Vail P. Insect pathogens as biological control agents: Do they have a future? Biological Control.
[20] Ruiu L. Insect pathogenic bacteria in integrated pest management. Insects. 2015; 6:352-367.
[21] Biological Products Industry Alliance.http://www.bpia.org/microbial-biopesticides/
[22] State of California's Department of Pesticide Regulation.http://www.cdpr.ca.gov/
[23] Jurat-Fuentes JL, Jackson TA. Bacterial entomopathogens. In: Vega FE and Kaya HK editors. Insect pathology. San Francisco: Academic Press; 2012. p. 265-304.
[24] Palma L, Muñoz D, Berry C, Murillo J, Caballero P. *Bacillus thuringiensis* toxins: An overview of their biocidal activity. Toxins (Basel).2014; 6: 3296–3325.
[25] Vega FE, Meyling NV, Luangsa-ard JJ, Blackwell M. 2012. Fungal Entomopathogens. In: Vega FE and Kaya HK editors. Insect pathology. San Francisco: Academic Press; 2012. p. 171-206.
[26] Mascarin GM, Jaronski ST. The production and uses of Beauveriabassiana as a microbial insecticide.World Journal of Microbiology & Biotechnology.2016; 32:177.
[27] Ahuja I, Kissen R, Bones AM.Phytoalexins in defense against pathogens.Trends in Plant Sciences.2012;17:73-90.
[28] War AR, Paulraj MG, Ahmad T, et al. Mechanisms of plant defense against insect herbivores. Plant Signaling&Behavior. 2012; 7(10):1306-1320.
[29] Tehri K, Singh N. The role of botanicals as green pesticides in integrated mosquito management – A review. International Journal of Mosquito Research 2015; 2 (1): 18-23.
[30] Isman MB. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world.Annual Review of Entomology.2006; 51:45-66.
[31] Enan E. Insecticidal activity of essential oils: octopaminergic sites of action. Comparative Biochemistry and Physiology. 2001; 130C:325–337.
[32] Tripathi AK, Upadhyay S, Bhuiyan M, Bhattacharya PR. A review on prospects of essential oils as biopesticide in insect-pest management.Journal of Pharmacognosy and Phytotherapy.2009; 1:052-063.
[33] El-Hosary RA. Evaluation of some essential oils against *Sesamiacretica* Led.under field conditions. Journal of American Science.2011; 7:563–568.
[34] Isman MB. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world.Annual Review of Entomology.2006; 51:45–66.
[35] Boursier CM, Bosco D, Coulibaly A, Negre M. Are traditional neem extract preparations as efficient as a commercial formulation of azadirachtin A? Crop Protection. 2011; 30: 318-322.
[36] Benelli G, Canale A, Toniolo C, Higuchi A, Murugan K, Pavela R, Nicoletti M. Neem (*Azadirachtaindica*): towards the ideal insecticide? Natural Product Research.2017; 31: 369-386.
[37] Perelló A, Gruhlke M, Slusarenko A. Effect of garlic extract on seed germination, seedling health, and vigour of pathogen-infested wheat. Journal of Plant Protection Research.2013; 53: 317-323.
[38] Perelló A, Noll U, Slusarenko AJ. In vitro efficacy of garlic extract to control fungal pathogens of wheat.Journal of Medicinal Plants Research.2013; 7: 1809-1817.
[39] Chiasson H, Vincent C, Bostanian NJ. Insecticidal properties of a Chenopodium-based botanical.Journal of Economic Entomology.2004; 97:1378-83.
[40] Kokanova-Nedialkova Z, Nedialkov PT, Nikolov SD. The genus chenopodium: Phytochemistry, ethnopharmacology and pharmacology. Pharmacognosy Reviews. 2009; 3: 280-306.
[41] Marcic D, Peric P, Prijovic M, Ogurlic I. Field and greenhouse evaluation of rapeseed spray oil against spider mites, green peach aphid and pear psylla in Serbia. Bulletin of Insectology.2009; 62: 159-167.
[42] Yu Q, Tsao R, Chiba M, Potter J. Elucidation of the nematicidal activity of bran and seed meal of oriental mustard (*Brassicajuncea*) under controlled conditions. Journal of Food, Agriculture & Environment.2007; 5: 374-379.
[43] Hopkins RJ, van Dam NM, van Loon JJ. Role of glucosinolates in insect-plant relationships and multitrophic interactions.Annual Review of Entomology.2009; 54: 57–83.
[44] Katsuda Y. Progress and future of pyrethroids.Topics in Current Chemistry.2012; 314: 1-30.
[45] Walters JK, Boswell LE, Green MK, Heumann MA, Karam LE, Morrissey BF, Waltz JE. Pyrethrin and Pyrethroid Illnesses in the Pacific Northwest: A Five-Year Review. Public Health Reports.2009; 124: 149–159.
[46] Hernández-Morales A, Arvizu-Gómez JL, Carranza-Álvarez C, Gómez-Luna BE, Alvarado-Sánchez B, Ramírez-Chávez E, Molina-Torres J. Larvicidal activity of affinin and its derived amides from *Heliopsislongipes* A. Gray Blake against *Anopheles albimanus* and *Aedesaegypti*. Journal of Asia-Pacific Entomology 18 (2015) 227–231.
[47] Molina-Torres J, Cabrera CJS, Armenta-Salinas C, Ramírez-Chávez E. Fungistatic and bacteriostatic activities of alkamides from *Heliopsislongipes* roots: Affinin and reduced amides. Journal Agricultural Food Chemistry.2004; 52: 4700–4704.
[48] Cantrell CL, Dayan FE, Duke SO. Natural products as sources for new pesticides.Journal of Natural Products.2012; 75:1231-1242.
[49] Fabricant DS, Farnsworth NR. The value of plants used in traditional medicine for drug discovery. Environmental Health Perspectives.2001; 109: 69–75.
[50] Lahlou M. The success of natural products in drug discovery.Pharmacology & Pharmacy.2013; 4:17-31.

[51] Wang L, Weller CL. Recent advances in extraction of nutraceuticals from plants. Trends in Food Science & Technology. 2006; 17: 300−312.
[52] Azmir J, Zaidul ISM, Rahman MM, Sharif KM, Mohamed A, Sahena F, Jahurul MHA, Ghafoor K, Norulaini NAN, Omar AKM. Techniques for extraction of bioactive compounds from plant materials: A review. Journal Food Engineering. 2013:117:426–436.
[53] Pino O, Sánchez Y, Rojas MM. Plant secondary metabolites as an alternative in pest management. I: Background, research approaches and trends. RevistaProtección Vegetal. 2013. 28: 81-94.
[54] Ibarra A, Cases J, Bily A, He K, Bai N, Roller M, Coussaert A, Ripoll C. Importance of extract standardization and *in vitro*/*ex vivo* assay selection for the evaluation of antioxidant activity of botanicals: A case study on three *rosmarinusofficinalis* L. extracts. Journal of Medicinal Food.2010; 13:1167–1175.
[55] Khan MSA, Ahmad I, Cameotra SS. Quality assessment of herbal drugs and medicinal plant products. In: Hostettmann EK, Marston A, Stuppner H, Chen S, editors. Handbook of chemical and biological plant analytical methods. Chichester, UK: John Wiley & sons; 2014. p. 1039-1057.
[56] Leggett M, Leland J, Kellar K, Epp B. Formulation of microbial biocontrol agents – an industrial perspective. Canadian Journal of Plant Pathology.2011; 33: 101-107.
[57] Arora NK, Mehnaz S, Balestrini R, editors. Bioformulations: for Sustainable Agriculture. India: Springer; 2016.
[58] Olson S. An analysis of the biopesticide market now and where it is going. Outlooks on Pest Management.2015; 26: 203-206.
[59] Tangtrakulwanich K, Reddy GVP. Development of insect resistance to plant biopesticides: an overview. In: Singh D editor. Advances in Plant Biopesticides. New Delhi: Springer; 2014. p. 47–62.

How to perform properly statistical analysis on food data? An e-learning tool: Advanced Statistics in Natural Sciences and Technologies

T. Eftimov[1,2], **P. Korošec**[1,3], **D. Potočnik**[2,4], **N. Ogrinc**[2,4], **D. Heath**[4] and **B. Koroušić Seljak**[1,2]

[1] Computer Systems Department, Jožef Stefan Institute, Jamova cesta 39, 1000, Ljubljana, Slovenia
[2] Jožef Stefan International Postgraduate School, Jamova cesta 39, 1000, Ljubljana, Slovenia
[3] Faculty of Mathematics, Natural Science and Information Technologies, Glagoljaška ulica 8, 6000 Koper, Slovenia
[4] Department of Environmental Sciences, Jožef Stefan Institute, Jamova cesta 39, 1000 Ljubljana, Slovenia

Many scientists have problems and difficulties in making a statistical analysis of their data, which they need to interpret their results. One problem is that applying a statistical method requires knowledge of the conditions (assumptions) about the data that must be met in order to apply it. These initial conditions are not usually checked and researchers simply apply a statistical method, in most cases taken from a similar published work, which is unsuited to their data set. As a result, their conclusions can be incorrect. This kind of misunderstanding is all too common in the research community. To become familiar with statistical methods, we provide a short tutorial on how to perform statistical analysis of food data. The study uses authentic food data on fatty acid profiles and the isotope composition of milk samples. In addition, we present an in-house developed and freely available e-learning tool for advanced statistics in natural sciences and technologies that has the benefit of checking the required conditions of each statistical method and offering only those methods that are appropriate for analysing the experimental data.

Keywords: statistical analysis; e-learning tool; food data analysis

1. Introduction

Many researchers have problems and difficulties performing a statistical analysis of their data, which is often crucial in interpreting their results. The problems appear because each statistical method has some conditions (assumptions) about the data that must be satisfied in order to apply the test [1]. Similarly, in food research, as in other research domains [2], researchers do not check for these conditions and simply apply a statistical method based on one used in a similar study. As a result, their conclusions can be incorrect. In addition, authors often do not provide the necessary information on the required conditions for selecting an appropriate statistical method. For example, to compare data from different data sets, two commonly used statistical tests are the paired t-test (if a comparison is made between two data sets) [3] and ANOVA (if a comparison is made between three or more data sets) [4]. The problem is that these tests are used even when the conditions for their safe use are not satisfied i.e., checking for normality, homoscedasticity (equality of variances) and independence. If the required conditions are not satisfied, an analyst will need to apply an alternative version of these tests. In many cases, calculating the $(1-\alpha)100\%$ confidence intervals [5] usually assumes that the data is normally distributed, when, in reality, the experimental data does not follow a normal distribution.

2. Statistical tutorial

To become familiar with statistical methods, we provide a short tutorial on how to perform a statistical analysis of food data. The tutorial is focused on descriptive statistics, hypothesis testing, and confidence intervals.

2.1 Population, representative data samples, and data types

A *population* is a set of similar items or events, which are of interest for some question or experiment, while a *representative data sample* is a set of data collected and/or selected from a statistical population by a defined procedure [1]. To see the difference between them, let us consider that a researcher is interested in how many people drink milk with breakfast in Slovenia. In this example, the population contains each person who lives in Slovenia. It would be unrealistic for us to ask each individual about his or her milk drinking habits, so instead we need a representative sample of people. The information obtained from the representative data sample allows a researcher to develop hypotheses about the larger population. In this example, let us consider that the researcher is a vegan and randomly selects a sample in which many of the participants are his or her friends many of whom are vegans. In this case, the number of people who drink milk with their breakfast will be lower when in reality the number is larger. This is an example of sample selection bias. In order to have a representative data sample, the researcher needs to be unbiased in their selection i.e., there are no outside factors influencing sample selection.
Once a representative data sample is selected, it becomes important to know what type of data has been collected [1]. There are two types of data: *qualitative* (categorical) and *quantitative* (numeric). Each type is split into two sub-types:

ordinal and *nominal* for qualitative data, and *discrete* and *continuous* for quantitative data. Ordinal data is categorical, where the values have natural, ordered categories and the distances between the categories are not known. For example, a question from a food questionnaire might ask: "How often do you eat fish during the week?". Possible answers include "Never", "one to three times per week", "four to six times per week", or "Every day". In this case, the answers are examples of ordinal data. Nominal data is also a categorical data, but in this case there is no natural order between the categories. Examples include eye colour, gender, and the region where one lives. In contrast to qualitative data, quantitative data is numerical. Discrete data can only take on a finite number of numeric data, while continuous data can take on an infinite number of possible values. Let us assume that a researcher is interested in the weight of participants in the data sample. The weight of each participant is stored as a continuous variable. However, he/she can split the participants into four groups according to their weight: (1) less than 50 kg, (2) 50 kg - 65 kg, (3) 66 kg - 80 kg, and (4) greater than 80 kg. In this case, weight is a discrete variable because it can take only one value from the four weight groups. This is an example of discretization, where a continuous variable is transformed into a discrete variable.

2.2 Descriptive statistics and inferential statistics

The next step is to apply statistics to the representative data sample. There are two different statistical analyses that we can apply: *descriptive statistics* and *inferential statistics* [6]. Descriptive statistics summarizes data from a sample using measures, while inferential statistics draws conclusions from the data set subject to random variation.

Descriptive statistics includes a distribution of single variable, measures of *central tendency*, which include the *mean*, *median*, and *mode*, and measures of *variability*, which include *standard deviation*, *variance*, and the *minimum* and *maximum* value. The distribution is a summary of the frequency of individual values or range of values for a variable. It represents every value of the variable and the number of how many times the value appears in the data sample. The central tendency of a distribution is an estimate of the "centre" of a distribution. The mean (average) is the most commonly used measure for central tendency and in order to compute it we sum all the values in our data sample and then divide the sum by the sample size (number of values in the data sample). The median is the value that separates the higher half of a data sample from the lower half, or it can be assumed as the "middle" value of an ordered data sample. The mode is the value from a data sample that appears with the highest frequency. The variability of a distribution refers to the spread of the data values around the central tendency. The minimum and the maximum value are the minimum and the maximum value that appear in the data sample. The standard deviation is used to quantify the amount of variation or dispersion of a set of data values. A low standard deviation indicates that the data values are close to the mean, while a high standard deviation indicates that the data values are spread out over a wider range of values. The variance is the square of the standard deviation.

Inferential statistics arise out of the fact that sampling naturally incurs sampling error and thus a sample is not expected perfectly to represent the population. The methods of inferential statistics include estimation of distribution parameters and the testing of statistical hypotheses.

When we talk about statistical analysis, it is also important to distinguish between *univariate*, *bivariate* and *multivariate* statistical analysis. Univariate analysis works when the data sample has only one variable. It does not deal with relationships and the major purpose is to describe the data. Bivariate analysis involves analysing two variables in order to find a relationship between them. Bivariate analysis is a special case of multivariate analysis when multiple relations between multiple variables are analysed. To explain better the difference between them, let us assume that the researcher is interested in testing whether or not consulting with a nutritionist can decrease a person's calorie intake. For this purpose, a researcher works with participants who consult with a nutritionist each day after work. The participants form two groups: one group that has daily consultations and a control group who do not receive any nutritional advice. After a week, let us assume that the researcher discovered that those who consult with a nutritionist have decreased their calorie intake for 40% over the control group. This is an example of univariate inferential statistics because it analysed the relationship between one independent variable (consulting with a nutritionist) with a single dependent variable (calorie intake). If a researcher is interested in the relationship between the times a person consulted a nutritionist and calorie intake over the week, then he/she needs to perform a bivariate analysis. If the researcher is interested to adding another variable i.e., how many times a person visited a gym, to determine the effectiveness of visiting a nutritionist and exercise, on two groups of people (20-40 yrs., and 41-60 yrs.), then he/she needs to perform multivariate analysis to find the relationships between all of the variables.

2.3 Hypothesis testing

One of the most commonly used approaches for testing relationships between two or more data samples is to use *hypothesis testing* [7]. Hypothesis testing is a procedure in which sample(s) data is employed to evaluate a hypothesis. The procedure involves two hypotheses: a *null hypothesis* (H_0) and an *alternative hypothesis* (H_A). The null hypothesis is a statement of "no effect" or "no difference", while the alternative hypothesis is a statement indicating the presence of an effect or a difference. We can define hypotheses about a single population or about the relationship between two or more populations e.g., between either the means or variances of a single, two or multiple distributions or even about the whole distribution.

After the data is collected, the next step is to apply hypothesis testing in order to evaluate the data using an appropriate *statistical test*. Each statistical test has a test statistic that has some distribution. A *test statistic* is a mathematical formula used to obtain a value using the collected data sample(s). Then the obtained value is compared with a value from a special table that contains information about the distribution of the test statistic. Such tables contain extreme values of the test statistic that are highly unlikely to occur if the null hypothesis is true. In order to obtain a value from such a table, a researcher first needs to specify a *level of significance* (α). The significance level is a probability threshold below which the null hypothesis will be rejected. Instead of specifying the significance level, a *p-value* can be used, which is the smallest level of significance that results in the rejection of the null hypothesis. The smaller p-value indicates that the null hypothesis is rejected, while a larger p-value indicates that the null hypothesis is not rejected.

In reality, the null hypothesis can be either true or false, and the result of a statistical test can be that the null hypothesis is either rejected or is not rejected. When performing hypothesis testing, two types of errors can occur: *type* I and *type* II. A *type I* error is the probability of rejecting the null hypothesis when it is actually true, while a *type II* error is the probability of not rejecting the null hypothesis when it is actually false. The probability of a type I error is the level of significance (α), so before the study we usually assign it a small value (e.g., 0.05, 0.01) because researchers do not want to have a type I error. The probability of type II error is marked as β and it is related to the *power of a statistical test*. The power is the probability that it will reject a false null hypothesis, or power = 1- β.

Power analysis is an important aspect in experimental design [8]. It allows researchers to determine the sample size required to detect an effect of a given size with a given degree of confidence. It allows researchers to find the probability of detecting an effect of a given size with a given significance level, under sample size constraints. If the probability is very low then researchers can change the sample size of the experiment.

The next step is to select an appropriate statistical test. There exist two types of tests: *parametric* and *nonparametric*. In order to distinguish between, we must check the conditions for the safe use of a parametric. These conditions include checking for *independence*, *normality of the data*, and *homoscedasticity of the variances*. Statistically, two events are independent if the occurrence of one does not influence the probability of the other occurring. Normality indicates that the data is normally distributed, which we can check by using statistical tests such as the Kolmogorov-Smirnov [9], Anderson-Darling [10], Shapiro-Wilk [11], and D'Agostino-Pearson test [12]. The result from a statistical test can be graphically proven using histograms or Q-Q plots (quantile-quantile). In probability, quantiles are cut points dividing the range of a probability distribution into intervals with equal probabilities. The homoscedasticity of variances indicates the hypothesis of equality of variances (homogeneity of variances). The Levene's test [13] can be used to check the homoscedasticity of the variances. If the required conditions for the safe use of parametric tests are satisfied, we must use a parametric test because it has higher power then a nonparametric test, otherwise we must select a nonparametric test.

Additionally to conditions for the safe use of parametric tests, other parameters that are also related to the selection of an appropriate statistical test is the number of data samples that need to be compared (two or more then two) and if the data samples are *paired* or *unpaired*. Paired samples (also called dependent samples) [1] are samples in which natural or matched couplings occur. So in the data sample each data value in one sample is uniquely paired to a data value in the second sample. Examples of paired samples are found in food questionnaires. Let us suppose that researchers are interested if there is a difference between two populations (males and females) according to the food questionnaire. In this case the obtained result of the male population for the first question is paired with the obtained result of the female population for the same question, and so on. The choice between paired and unpaired samples depends on experimental design, and researchers need to be aware of this when designing their experiment.

The first step for performing hypothesis testing is to check all the conditions involving the data sample(s), and then to decide which statistical test is the most appropriate. Table 1 presents the different statistical test classified according to the conditions that must be met.

If a researcher wishes to compare more than two data samples, first he/she needs to check the conditions for independence, normality of data, and homoscedasticity of variances. If they are satisfied, then he/she needs to determine if the samples are either paired or unpaired. If he/she has unpaired samples, an appropriate statistical test to use is the one-way ANOVA. If at least one of the conditions for the safe use of a parametric test is not satisfied, then he/she needs to select a nonparametric alternative such as the Kruskal-Wallis test.

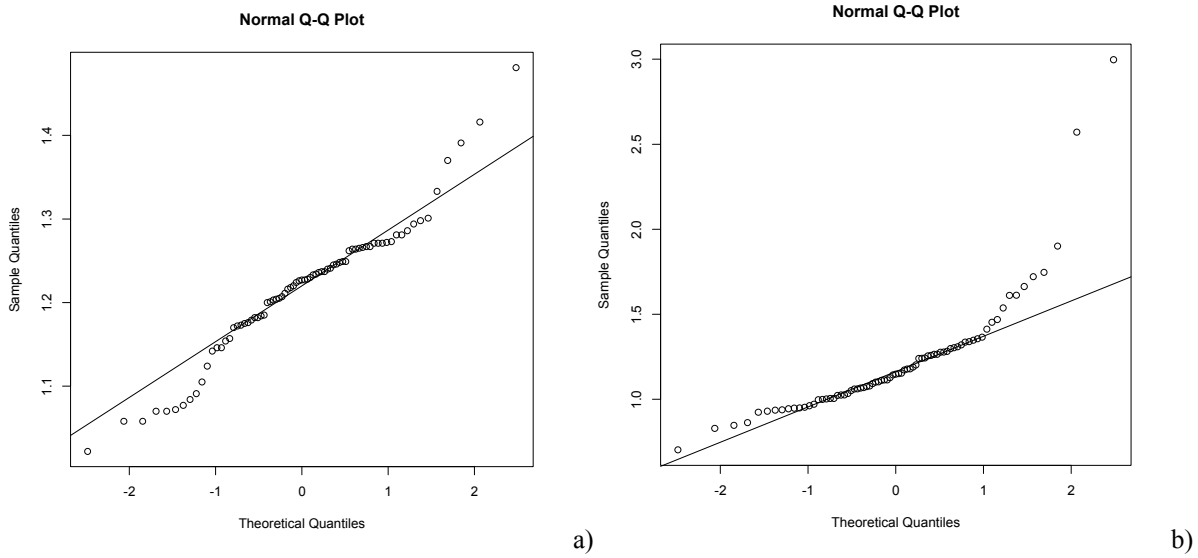
Table 1 Classification of different statistical test.

	Two data samples	**More then two data samples**
Parametric	t-test (unpaired) [14] Paired t-test (paired) [3]	One-way ANOVA (unpaired) [4] Repeated-measures ANOVA (paired) [15]
Nonparametric	Mann-Whitney U (unpaired) [16] Wilcoxon signed-rank (paired) [18]	Kruskal-Wallis (unpaired) [17] Friedman, Friedman-aligned, Iman-Davenport (paired) [19]

2.3.1 Example of comparing two data samples

This example involves comparing two data samples. The data is authentic food data and represents the fatty acid profile of Slovenian milk samples. Fatty acids composition in milk can be significantly altered by the nutrition of the dairy cows and their metabolisms and thus can provide information about the provenance of milk and dairy products. One batch of samples contains the fatty acid profile of 77 milk samples collected in 2013, while the other fatty acid profile of 77 samples collected in 2014. Comparing both data samples, we are interested to test if there is difference between the fatty acid profiles of milk sampled in 2013 and 2014.

First, we started by checking the required conditions for the safe use of the parametric tests. The independence is satisfied because the milk samples are independent. The normality condition is checked using the Shapiro-Wilk test, for which the significance level is set to 0.05. The p-value using this test with the data from 2013 is 0.02, while the p-value obtained with the data from 2014 is 0.00. In both cases the p-values are smaller then the significance level, which indicates that the data from both samples is not normally distributed. Figure 1 shows a graphical representation of the data from both data samples using normal quantile-quantile (Q-Q) plots. If the data is normally distributed, the values in the plot will lie on a straight diagonal line, which is not the case for our both samples.



a) b)

Fig. 1 Normal Q-Q (quantile-quantile) plots for the data samples from a) 2013 and b) 2014.

Using the result form the Shapiro-Wilk test and the normal Q-Q plots, we see that normality condition is not satisfied, so we do not need to check the homoscedasticity of variances, because we can not select a parametric test, and instead we need to select a nonparametric test. However, using the Levene's test, the p-value is 0.00, which is smaller than 0.05, and there is no homogeneity of variances. Because there are two data samples that are unpaired, an appropriate test is the Mann-Whitney U test. Using it, the p-value is 0.04, which is smaller than 0.05, the significance level set prior to analysis, and the null hypothesis is rejected, so there is a significant statistical difference between the fatty acid profiles measured between two different years.

2.3.2 Example of comparing three data samples

This example involves comparing three data samples. The data presented is authentic food data on the fatty acid profile (different from the fatty acid profile in the first example) in Slovenian milk samples. The first sample contains the fatty acid profile measured in 77 milk samples from 2012, the second contains the same fatty acid profile measured in 77 samples form 2013, while the third sample contains the same fatty acid profile measured in 77 samples from 2014. Comparing the three data samples, we are interested to test if there is a difference between the fatty acid profile of milk collected in 2012, 2013 and 2014.

As in the case of comparing two data samples, we started by checking the required conditions for the safe use of parametric tests. In this case independence is satisfied because the milk samples are independent. To check for

normality we use the Shapiro-Wilk test, for which the significance level is set to 0.05. The p-value obtained for the sample form 2012 is 0.00, the p-value for the sample from 2013 is 0.00, and the p-value for the sample from 2014 is 0.00. All p-values are smaller than 0.05, so the normality condition is not satisfied. Since, therefore, we cannot use a parametric test we must select a nonparametric one. The p-value for homoscedasticity of variances is 0.03 and is smaller than our selected significance level 0.05, which means that we reject the null hypothesis and there is no homogeneity of variances. Because there are three data samples that are unpaired, an appropriate statistical test is the Kruskal-Wallis test. Using it, the p-value is 0.87, which is greater than 0.05 and we do not reject the null hypothesis, so there is no significant statistical difference between the fatty acid profiles of milk collected from the three different years. If there is a significant statistical difference and we are interested to see from which pairs of data samples this difference appears, we need to continue with a post-hoc statistical test appropriate for the Kruskal-Wallis test.

2.4 Confidence interval

In some experiments, it can happen that the distribution of the data collected in a data sample is known, but the parameters that describe the distribution are unknown and must be estimated from the data sample. For example, let us assume that a researcher has data that is normally distributed, but he/she does not know the mean and the standard deviation of the normal distribution. One way to estimate them is to use the (1-α)100% *confidence interval* (CI) [5]. A CI is a type of interval estimate of an unknown parameter. It is calculated using the collected experimental data and it potentially includes the unobservable parameter of interest. The parameter that indicates how frequently the interval contains the true parameter is the confidence level (α). For example, a 95% CI indicates there is 95% probability that the calculated CI contains the true value of the population parameter, but it does not mean that for a given interval there is a 95% probability that the population parameter lies within the interval.

The most common approach for calculating the CI is to use the sigma, two-sigma, three-sigma rule of thumb (68-95-99.7 rule) [20]. However, we can only apply this rule when the data is normally distributed. So before using it, we must first check to see if the data is normally distributed. In reality, many papers include CIs calculated using this rule even when the normality condition is not satisfied resulting in incorrect results. So the question that arises here is how to calculate the CI when the normality condition is not satisfied. The answer is to use bootstrap confidence intervals. Bootstrapping involves random sampling with replacement, which means randomly selecting a subset of values from the data sample in which a value may appear multiple times in the selected subset [21]. We use it to estimate parameters by measuring the properties when sampling form an approximating distribution, which in practice is the empirical distribution function of the observed data. In the case where we can assume that a set of values are independent and identically distributed, bootstrapping can be performed using a number of resamples with replacement of the observed data sample, which have equal size to the observed data sample.

2.4.1 Example of calculating confidence interval

This example involves finding a 95% CI for the parameters: means and variance, using a data sample that consists of the fatty acid profiles measured in milk samples (77) from 2014. First, we check for normality using the Shapiro-Wilk test at a significance level 0.05. The p-value is 0.00, so it indicates that the data is not normally distributed, meaning we cannot use the two-sigma rule and instead use bootstrapping. Using bootstrapping the CI for the mean is [1.22; 1.41] and the CI for the variance is [0.10; 0.47]. The calculated CI for the mean parameter indicates that there is 95% probability that the calculated CI contains the true value of the mean, while the calculated CI for the variance indicates that there is 95% probability that the calculated CI contains the true variance.

3. An e-learning tool: Advanced Statistics in Natural Sciences and Technologies

3.1 An in-house developed open-source e-learning tool

We developed an in-house freely available e-learning tool for advanced statistics in natural sciences and technologies, which covers the statistical tutorial outlined in this chapter. At present, it contains methods for linear regression, which is an approach for modelling the relationship between a scalar dependent variable and one or more independent variables [22] and principal component analysis (PCA), which is an approach to convert a set of observations of correlated variables into a set of values of linearly uncorrelated variables [23]. The benefit of the e-learning tool is that it checks for the required conditions of each statistical method and offers only those methods that are appropriate for analysing the experimental data. The e-learning tool is available free to use on registration at http://ws.ijs.si/statTool/. The tool includes “Basic statistics”, “Hypothesis testing”, “Confidence interval”, “Regression analysis”, and “Dimensionality reduction”. There is also a template for organizing the data, which must be a comma-separated values file (csv). After uploading the data, the e-learning tool checks the data and will give an error message if either the decimal values are represented by a decimal point, the data contains any character values or if any values missing. Figure 2 shows the registration tab of the e-learning tool.

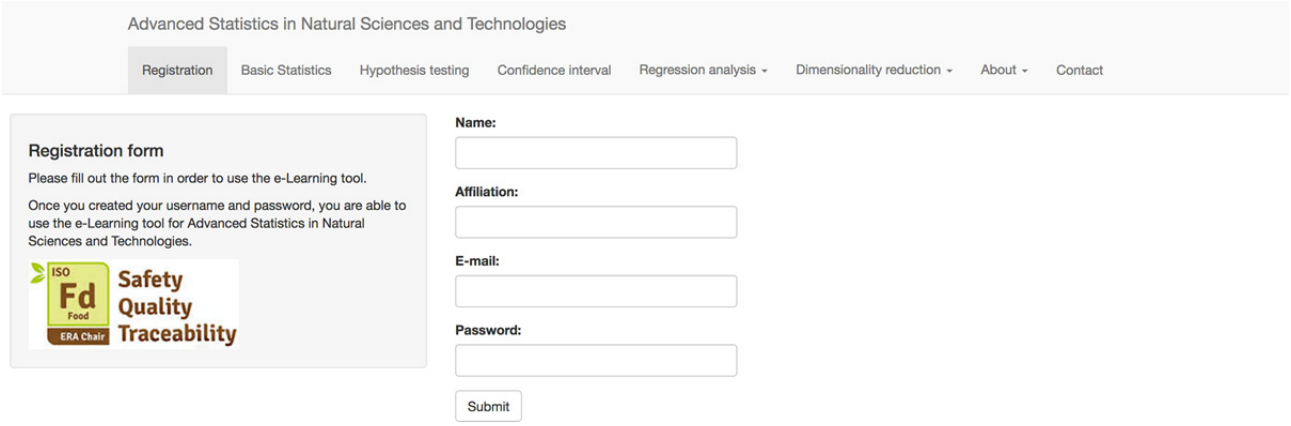


Fig. 2 The registration part of the e-learning tool.

The idea for such a tool came about after asking a sample of 28 researchers (master and doctoral level) who work in food analysis to select a method to do the following: i) compare two data samples, ii) compare three or more data samples, and iii) calculate a confidence interval. In the first case, 36.36% selected a paired-t test, while 45.46% did not know which statistical test to use. Only 18.18% chose the correct answer, which was to first check the conditions necessary for each method based on their data and then decide on the method to use. In the second case, 81.82% selected to use ANOVA, while again only 18.18% chose to check the conditions of each method before selecting which statistical method to use. Finally, when calculating the confidence interval just over half of the participants (54.54%) selected the standard formula that requires normally distributed data, even though their data is not normally distributed, i.e., they did not know which formula to use. In this case, none of the participants chose to check first the conditions of the data. It is clear that only a small percentage of the participants know how to perform correctly a statistical analysis.

A presentation on statistical analysis was presented to participants of the ISO-FOOD spring school on the use of isotopes in food organized from 4th to 7th April 2017 at Jožef Stefan Institute, Ljubljana, Slovenia [24]. After an introductory presentation, the participants (master and doctoral level researchers) where asked to complete the same three tasks as earlier. After dividing the participants into three groups they were given thirty minutes to perform one of the three examples using a statistical software package in which they were experienced. The data used for the tasks is available at (http://cs.ijs.si/opendata/DataSets.zip). Each group then presented their results. The group that worked on task one, began by using Excel, but switched to RStudio [25] since Excel was taking too much time. First, they checked the required conditions for the safe use of a parametric test and because the tests were not satisfied, they selected an appropriate nonparametric test, which in their case was the Mann-Whitney U test, whereas before the presentation, the majority of opinion was to use a paired-t test. The second group chose to compare the three data samples using three different pairwise comparisons by comparing the variances of the data samples using the Fisher test [26]. However, this is not correct because these pairwise comparisons are independent and combined independent p-values need to be additionally calculated to control the family wise error (FWER), which is the probability of making type I errors when performing multiple hypotheses tests [27]. The group also explained that they performed multiple pairwise comparisons because they could not find the ANOVA function in Excel. However, the appropriate statistical test in this example was Kruskal-Wallis test. The third group, which worked on calculating CI, also used Excel using the equations for normally distributed data despite this not being the case in the test data. Afterwards, they switched from Excel to SPSS [28], and performed the same experiment reporting the bootstrapping confidence intervals, which were the correct results for this experiment. Prior to the presentation, when asked, the suggestion was to use the sigma, two-sigma, three-sigma rule.

Each group was then set the task of reviewing three scientific papers published in a journal with high impact factor and then to judge if the statistical analysis used is correct and what if any information relating to statistics is missing. All groups agreed that in the three reviewed papers the authors only said that they used the t-test to compare two data samples and a one-way ANOVA to compare more then two data samples. They did not provide the results for checking the required conditions for the safe use of parametric tests, so it is not clear how they selected the statistical test or if they also followed a similar study. In addition, the CIs were reported using the sigma, two-sigma, three-sigma rule without first checking if the data was normally distributed or not.

Finally, after a short presentation of the e-learning tool each group repeated each of the three tasks. All three groups needed only a few minutes to perform the three examples. For the first example, they needed only to upload the test data, and to indicate if they have paired or unpaired samples. The e-learning tool checks all other conditions and offers them with the most suitable statistical test, in this case the Mann-Whitney U test. For the second example, the tool offered only the Kruskal-Wallis test (Fig. 3), which meant that they did not have to check for either normality or homoscedasticity of the variances. Finally for the third task, the participants needed to specify only which parameter they want to calculate the CI and the tool provides the CI together with the method used to obtain it.

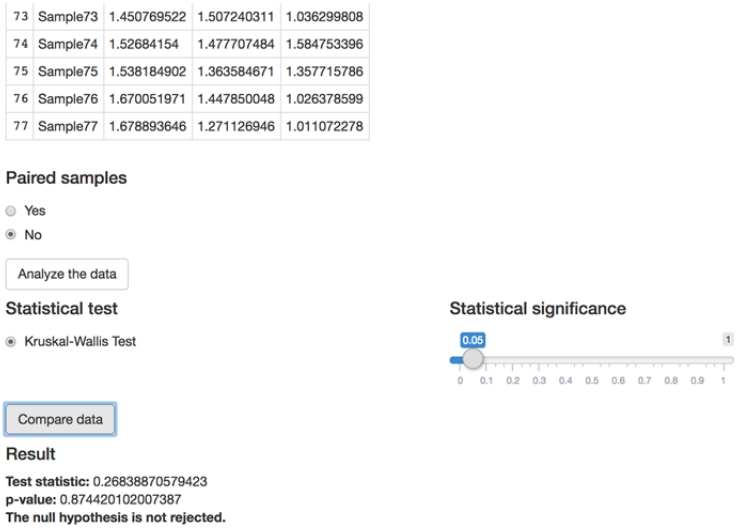


Fig. 3 The result from the e-learning tool when used to compare three data samples. The tool checks the required conditions for the safe use of parametric tests, which in this case are not satisfied, and offers the user the Kruskal-Wallis test, which is a nonparametric test.

4. Conclusion

In this chapter, we present a statistical tutorial of how to perform properly statistical analysis on food data. In addition, an e-learning tool is presented. The e-learning tool automatically checks the conditions for each method and offers only those methods that are appropriate for the data. Overall, the e-learning tool not only reduces the time needed to perform a statistical analysis but importantly, it can help in interpreting results and increase awareness of using an inappropriate statistical method. In the future, we plan to upgrade the e-learning tool with a power analysis to help researchers to select a relevant sample size for their experiments.

Acknowledgements This work is supported by the project ISO-FOOD, which received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement No. 621329 (2014-2019). The authors acknowledge the financial support from the Slovenian Research Agency for the project "The content of trans fats in foods and population intakes - public health implications" (research core funding No. L3-7538).

References

[1] Ostle B, Mensing RW. Statistics in research: Basic concepts and techniques for research workers. Ames, IA: Iowa State University Press.1975.
[2] Eftimov T, Korošec P, Koroušić Seljak B. Disadvantages of Statistical Comparison of Stochastic Optimization Algorithms. Proceedings of the Bioinspired Optimizaiton Methods and their Applications, BIOMA. 2016.
[3] Zimmerman DW, Teacher's corner: A note on interpretation of the paired-samples t test. Journal of Educational and Behavioral Statistics. 1997; 22.3:349-360.
[4] Stoline MR. The status of multiple comparisons: simultaneous estimation of all pairwise comparisons in one-way ANOVA designs. The American Statistician. 1981; 35.3: 134-141.
[5] Newcombe RG. Two-sided confidence intervals for the single proportion: comparison of seven methods. Statistics in medicine. 1998; 17.8: 857-872
[6] Lapin LL. Probability and statistics for modern engineering. Thomson Books/Cole. 1983.
[7] Winer BJ, Brown DR, Michels KM. Statistical principles in experimental design. New York: McGraw-Hill. 1971; 2.
[8] Erdfelder E, Faul F, Buchner A. GPOWER: A general power analysis program. Behavior research methods, instruments & computers. 1996; 28:1-11.
[9] Lilliefors HW. On the Kolmogorov-Smirnov test for normality with mean and variance unknown. Journal of the American statistical Association. 1967; 62.318:399-402.
[10] Pettit AN. Testing the normality of several independent samples using the Anderson-Darling statistic. Applied Statistics. 1997. 156-161.
[11] Shapiro SS, Wilk MB. An analysis of variance test for normality (complete samples). Biometrika. 1965; 52.3-4:591-611.
[12] Pearson ES, D'agostino RB, Bowman KO. Tests for departure from normality: Comparisons of powers. Biometrika. 1977;231-246.
[13] Schultz BB. Levene's test for relative variation. Systematic Biology. 1985; 34.4: 449-456.
[14] Cressie NAC, Whitford HJ. How to Use the Two Sample t-Test. Biomedical Journal. 1986; 28.2:131-148.
[15] Dien J, Santuzzi AM. 4 Application of Repeated Measures ANOVA to High-Density ERP. Event-related potentials: A methods handbook. 2004; 57-81.

[16] Mann HB, Whitney DR. On a test of whether one of two random variables is stochastically larger that the other. The annals of mathematical statistics. 1947; 50-60.
[17] Breslow N. A generalized Kruskal-Wallis test for comparing K samples subject to unequal patterns of censorship. Biometrica. 1970; 579-594.
[18] Conover WJ, Iman RL. Rank transformations as a bridge between parametric and nonparametric statistics. The American Statistician. 1981; 35.3:124-129.
[19] Derrac J, Garcia S, Molina D, Herrera F. A practical tutorial on the use of nonparametric statistical tests as a methodology for computing evolutionary and swarm intelligence algorithms. Swarm and Evolutionary Computation. 2011; 1.1:3-11.
[20] Kriegel HP, Kroger P, Schubert E, Zimek A. LoOP: local outlier probabilities. Proceedings of the 18th ACM conference on Information and knowledge management. 2009.
[21] Efron B, Tibshirani R. Bootstrap methods for standard errors, confidence intervals, and other measures of statistical accuracy. Statistical science. 1986; 54-75.
[22] Seber GAF, Lee AJ. Linear regression analysis. John Wiley & Sons. 2012; 936.
[23] Jolliffe I. Principal Component Analysis. John Wiley & Sons, Ltd. 2002.
[24] Era Chair for isotope techniques in food quality, safety and traceability – ISO-FOOD, http://isofood.eu/
[25] Team RStudio. RStudio: integrated development for R. R Studio, Inc., Boston, MA URL http://www.rstudio.com. 2015.
[26] Cochran WG. Approximate significance levels of the Behrens-Fisher test. Biometrics. 1964; 20.1:191-195.
[27] van der Laan MJ, Dudoit S, Pollard KS. Multiple testing. Part II. Step-down procedures for control of the family-wise error rate. Statistical applications in genetics and molecular biology. 2004; 3.1:1041.
[28] Green SB, Salkind NJ. Using SPSS for Windows and Macintosh: Analyzing and understanding data. Prentice Hall Press. 2010.

Integration of multi-omics data for biomarker identification of food safety and quality

G. Perrotta[1], **M. Donini**[2], **O.C. Demurtas**[2], **A. Mengoni**[3], **P. De Rossi**[2], **A. Del Fiore**[2], **M. Di Carli**[4], **L. Bianco**[1], **L. Daddiego**[1], **M. Sulli**[2], **L. Daroda**[2] and **A. Bevivino**[2,*]

[1] Department of Energetic Technologies, Bioenergy, Biorefinery and Green Chemistry Division, ENEA Trisaia Research Center, Rotondella, Italy
[2] Department of Sustainability and Productivity of Territorial Systems, Biotechnology and Agro-Industry Division, ENEA Casaccia Research Center, Rome, Italy
[3] Department of Biology, University of Florence, Florence, Italy
* Corresponding author: email: annamaria.bevivino@enea.it

Keywords: Omics; Data integration; System biology; Food safety; Food quality

In the last decades, consumers' interest in healthy, fresh and convenience foods has greatly increased. Due to globalization, food scenario is rapidly changing and moving from the consideration of food as a mere source of energy to a growing awareness on its importance for health and particularly in reducing the risk of diseases. The continuous advance in the field of molecular biology allowed setting up efficient and universal omics tools to address food safety and quality. In this review, we look at the current progress of applying omics technologies to identify biomarkers of food safety and quality. We consider the application of a multi-omics approach integrating proteomics, metabolomics, metagenomics, transcriptomics (via systems biology), in characterizing the composition of food products along the food supply chain. The combination of the above omics approaches allows us to define a sort of molecular labeling of food or biomarkers that are easily understandable by the operators involved in the food sector.

1. Introduction: the challenge of omics in the field of food safety and quality

The globalization of the food market has raised major concerns in terms of safety and quality of foods due to the worldwide increase in the movement of foodstuff and related raw materials worldwide as well as the shipments of multiple and processed ingredients from different parts of the globe [1]. Food chain, food safety, and food-processing sectors face new challenges due to the globalization and the continuous changes in the modern consumer preferences. In addition, the gradual increase in microbial resistance, changes in climate, and incorrect food handling remain a pending barrier for the efficient global food safety management [2]. An important aspect of food science is the need to trace food products along the entire food supply chain and to ensure all the safety, nutritional quality and acceptability issues related to of the delivered products. Although at present half of planet's population doesn't have access to a sufficiently nutritious diet [3], in the countries where economic growth is present there is a demand and an emerging trend for nutritiously and healthy foods, as well as a rising consumer concern for food safety and quality. In fact, food safety refers to all those hazards, whether chronic or acute, that may have a negative impact on the health of the consumer, while food quality mainly refers to all other attributes that influence a product's value to the consumer, including contamination, discoloration, off-odours, origin, colour, flavour, texture, and processing method of the food. In the past two decades, our ability to evaluate the food safety and quality has radically changed through the development of high-throughput, omics technologies [4] (Figure 1 and Table 1).

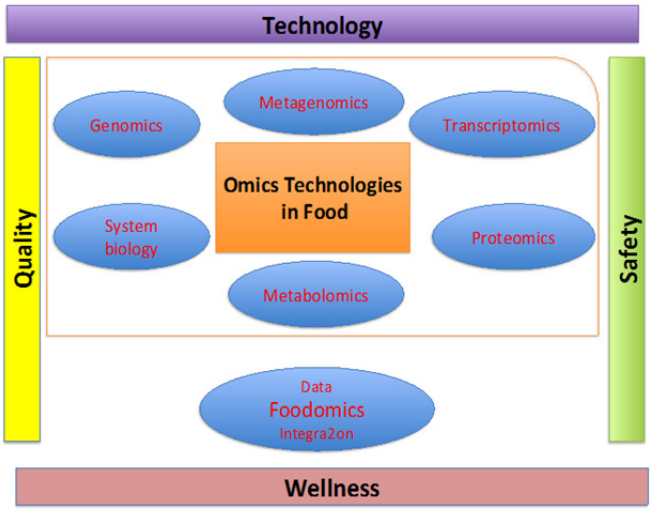


Fig. 1 A new comprehensive approach to food quality and safety assessment.

Table 1 The role of omics technologies for the detection of food quality and safety.

Omics approaches	Molecules of interest	Description	Approaches and technologies	References
Genomics	DNA	Genomics is the systematic study of the structure, function, and expression of all the genes in a cell/ organism/sample	16S rRNA gene clone library, Quantitative PCR, Whole genome sequencing	[5], [6], [7]
Metagenomics	DNA	Metagenomics is defined as the direct genetic analysis of genomes contained with a cell/organism/sample	16S rRNA gene sequencing, Shotgun sequencing	[8], [9], [10]
Transcriptomics	RNA	Transcriptomics is the study of the total mRNA in a cell organism/sample	DNA microarray, RNA sequencing	[11], [12]
Proteomics	Proteins	Proteomics is the large-scale study of proteins, including their structure and function, within a cell/ /organism/sample	Peptide mass fingerprinting (PMF), MS/MS, LC-MS, MS, DIGE technology	[13], [14]
Metabolomics	Metabolites	Metabolomics is the study of global metabolite profiles in a system (cell, tissue or organism) under a given set of conditions	Fourier Transform Infra-Red Spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR), Mass Spectroscopy (MS) and High Resolution Mass Spectrometry (HRMS)	[15], [16], [17]
System biology	All	System biology is the systematic study of complex interactions in biological systems	Integration models	[18], [19]
Foodomics	All	Foodomics is the comprehensive, high-throughput approach for the exploitation of food science in the light of an improvement of human nutrition	High-throughput approach in the field of food chemistry, analytical chemistry, biochemistry, microbiology, molecular biology, food technology, clinical sciences, and human	[2], [20]

The advancement in sequencing technologies and various 'omics' tools has impressively accelerated the research in this area, presenting several advantages over traditional approaches [21]. In fact, compared to traditional methods, omics technologies appear to combine the benefits of relative simplicity, sensitivity, the speed of generating information and analysis of foods at various levels. By utilizing omics technologies, researchers can comprehensively compare food products at a molecular level, by analyzing the protein/gene expression/microbiome/metabolite components and composition changes during processing, storage, and transport. The *omics* approach has revolutionized the study of food allowing to develop suitable biomarkers for addressing food quality, authenticity, and safety issues, as well as to correlate all food components to the individual diet and the health. To achieve this goal, researchers involved in modern food science need to work within multidisciplinary teams in order to be able to face the huge complexity of this task and to rationally handle the huge amount of data generated by omics technologies (Figure 1). The integration of different omics technologies and the use of bioinformatic and advanced computational tools are key factors in the system-level understanding of relevant genes, variants, pathways or metabolic functions characterizing the food products and to monitor critical points in the food production/manipulation chain and the processes in the food industry.

2. Genomics and Metagenomics

The most common way of determining the composition of food-associated microorganisms has been through culturing methods that are based on the isolation and cultivation of microorganisms before their identification and typing. Genomic analysis to identify isolates to the genus or species level is more reliable and has greater discrimination than phenotypic methods. Among the plethora of molecular identification and characterization technologies available to date, the Whole Genome Sequencing (WGS) represents a significant tool in the area of food safety and food technology developments. This technique is being adopted by regulatory agencies around the world to identify bacterial isolates from foods, due to advances in sequencing technology (Figure 2). The entire genome sequences of numerous foodborne pathogens have been determined and the rapid and accurate detection and identification of foodborne pathogens are possible due to the many useful sequence analysis tools and software programs available in public WGS databases [22]. There are several examples of using sequencing for solving the epidemiological source of

foodborne microbial outbreaks [23]. In addition, the identification of bacteria at strain level by means of molecular typing tools, such as pulse-field gel electrophoresis (PFGE), ribotyping, and PCR-based techniques, is important for the purposes of surveillance and outbreak investigation. Anyway, all the above molecular methods that permit to identify and type food microorganisms require the isolation and culturing of microorganisms. Cultivation of microorganisms poses several limits and potential problems, which can be overcome by nucleic acid-based detection methods [9] [24]. Those limits include: i) the impossibility to detect most of the foodborne viral pathogens, ii) the presence of pathogenic bacteria in the viable but not cultivable state (VBNC), the ambiguous identification of pathogens due to the lack of selective media, iv) the possible long times necessary for the growth of the microorganism.

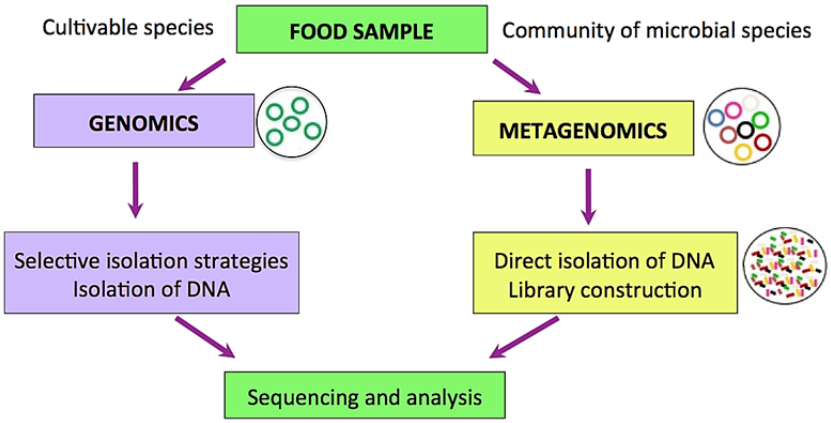


Fig. 2 Schematic representation of the differences between genomics and metagenomics.

Over recent decades, a great number of culture-independent methods have been developed that help overcome these problems; most of which have been used extensively in food systems [10]. Since the introduction of the DNA-based methods in microbial ecology, it became clear their potentiality in food microbiology, as a set of tools for fast and precise identification of target pathogenic organisms as well as for the detection of food quality [25]. Such DNA-based methodologies generally rely on specific DNA sequences (markers) that can be used for approving the quality and origin of raw ingredients. 16S ribosomal RNA (rRNA) gene sequences are commonly used to identify, quantify, and visualize microorganism populations in foods [26] because their genes consist of highly conserved domains interspersed with variable regions. Barcode sequencing of 16S rDNA performed on total microbial DNA from food samples enabled to evaluate the microbial diversity based on Operation Taxonomic Units (OTUs) composition as well as to assess their taxonomic status, and to further individuate biomarkers for their application in addressing quality, technology, authenticity, and safety issues. The application of specific DNA detection methods, as Real-Time PCR protocols, has been promoted and specific international agreements and standards have been released [27] [28]. In the last ten years, the advances in massive parallel sequencing or high throughput sequencing (HTS) technologies have opened a new perspective in food microbiology [29]. The development in metagenomic approach opens the way to previously unknown scenarios to detect microbial activities in microbes without requiring their cultivation since it gives the possibility to analyze the meta-community dynamics and to identify markers of food microbiota changes following the processing, treatments, transport and storage process. Among the most commonly reported HTS technologies are Illumina, where an efficient protocol for 16S rRNA gene-based analysis is the most widely used [30] but other technologies are on the market and used in microbial pathogen detection, as the 454 Roche pyrosequencing, the non-optical Ion Torrent device, and the single-molecule systems Nanopore-MinION and PacBio-SMRT (see for examples [31] [32] [33]). Those technologies may enable both amplicon-based sequencing (using 16S rRNA amplification primers targeting hypervariable regions) and untargeted metagenomic analyses (where sequence fragments from the virtually whole environmental DNA, including all the microbiome present in the sample, is reported). In this way, it is possible to detect the presence of specific microbial taxa and strains and the presence of functional genes in their genomes (e.g. toxin-encoding genes).

Metagenomics (i.e., the study of microbial communities sampled directly from their natural environment, without prior culturing) is currently applied primarily for the study of the microbiota composition from an ecology perspective (Figure 2). This technology offers a path to the study of their community structures, phylogenetic composition, species diversity, metabolic capacity, and functional diversity. In food science, it is essential to evaluate not only the species diversity of microbial communities but also to analyze how the species structures of those communities change over time and space.

Metagenomic sequencing can widen our perception of food microbiology, from a focus on a (small) panel of microbial taxa to an ecological interpretation of the food microbiome, as a “living” matter, where microbes interact each other and may possibly establish antagonistic interactions, which in turn, can limit the growth of undesirable strains [9]. Both the targeted one (“16S metagenomics”) and the untargeted one (“metagenomics *sensu stricto*”)

require several steps for data generation and processing. In particular, DNA must be extracted from the matrix under study and should be of enough quantity and quality for its successive PCR amplification (for targeted metagenomics) and, especially, for untargeted metagenomics. Moreover, DNA coming from the matrix (environmental DNA, eDNA) includes both the microbial DNA and the matrix (plant/animal DNA). Consequently, strategies should be used to reduce the amount of non-microbial DNA in the eDNA preparation or to select in the targeted metagenomics, microbial-specific target genomic regions or selective PCR primers (as for 16S rRNA, avoiding the amplification of plastid, hence plant-derived, 16S rRNA genes). Furthermore, depending on the level of sensitivity (i.e. the threshold of detection) the number of sequence reads per sample should be chosen in order to detect even low abundance taxa and to allow a good coverage of the microbial diversity in the sample. Finally, standardized procedures for sequence binning, operating taxonomic unit (OTU) attribution and assignment to microbial taxonomy must be selected after careful evaluation. This is especially critical for the database of taxonomic ribosomal references, as for instance Greengenes [34], RDP [35] or Silva [36], where different databases or different confidence thresholds may produce ambiguous microbial identifications.

However, from a food safety microbiology perspective, HTS will still require standardization of procedures for sample preparation, nucleic acid extraction, sequence data processing, etc. Moreover, HTS, tough their cost is decreased in the last years remain more expensive than Real-Time PCR in the case just a small number of targets has to be evaluated, and require more time from sample preparation to the bioinformatic data report.

3. Proteomics

In the last decade, we all witnessed a strong increase in the application of proteomics in the study, control, and validation of industrial processes of food products and in the assessment of food quality, safety, and traceability [14]. In particular, an enormous work has been done in the field of the meat, fish and dairy industry contributing for example to the identification of the specific biomarker for meat tenderness or traceability, fish flavour or to the evaluation of milk quality for the improvement of infant formulas and cheese making. In the case of proteomics applied to agriculture, a strong effort has been made in the assessment of the quality control of food product derived from transgenic crops, in the quality control processes of beer and wine industry and also in the characterization of the composition and quality of ready-to-eat vegetable products [37]. Another important issue that has been addressed by proteomics is food safety represented by the possible contamination by pathogenic microorganisms and microbial toxins, allergens, and toxic compounds. In this paragraph we will review the latest methodological approaches used in proteomics (gel-based and gel-free) such as comparative 2D electrophoresis, quantitative isotope labeling as well as the most recent and innovative label-free quantitative proteomics techniques, providing some examples of their application in food analysis (Figure 3).

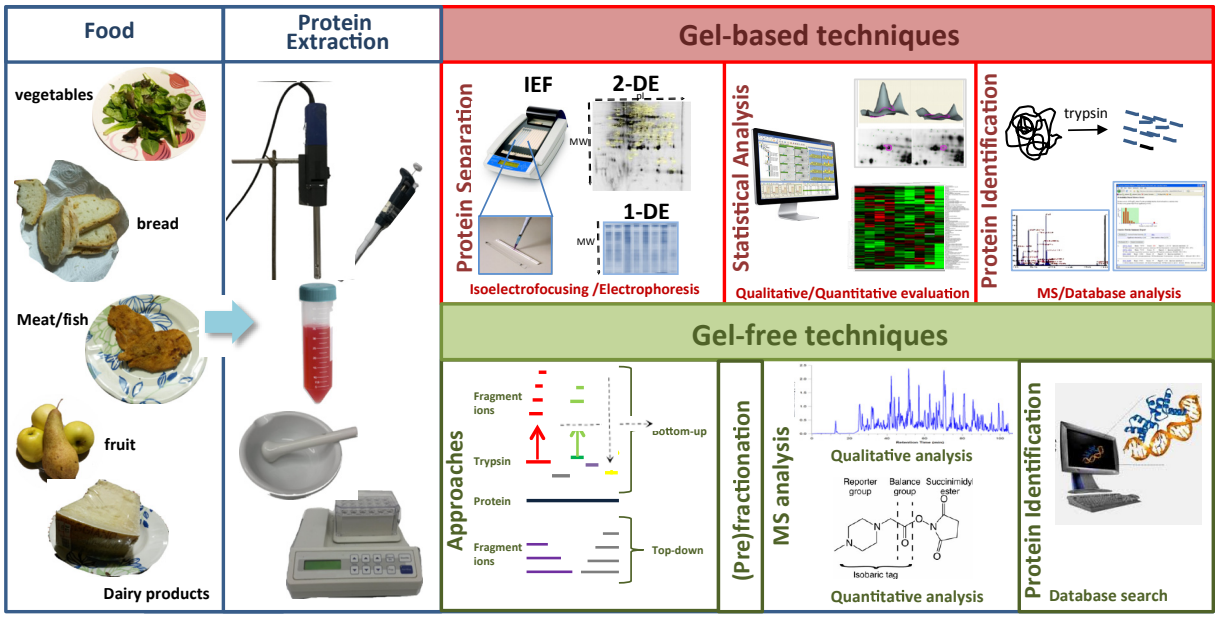


Fig. 3 Schematic representation of the proteomics analysis workflow using both gel-based and gel-free methodological approaches.

3.1 Gel-based approaches

The electrophoretic protein separation approach combined with MS analysis has become one of the most used methodologies in the food control, safety, and traceability. In fact, among gel-based techniques, two-dimensional gel electrophoresis (2-DE) has been widely used in the identification of quality markers of various foods, from meat and fish to vegetables, of biological or transgenic origin [38].

The major advantage of this technique is that 2-DE provides the highest protein-resolution capacity with a low-instrumentation cost. The typical workflow consists of the first phase of protein extraction from food sample (Figure 3). Then proteins are isolated by a bidimensional separation based on isoelectric focusing point and molecular weight on the SDS-polyacrylamide gel (PAGE). In the second phase after protein staining, the image analysis, using dedicated software, allows determining quantitative and qualitative variation, comparing the intensities of protein spots in different gels. In the third phase proteins from individually excised spots are enzymatically digested with trypsin, and the resulting peptides are analyzed by mass spectrometry to be finally identified by database searching [39].

The main limitations of this methodology are represented by the experimental variations that occur among gels. An advance in 2-DE is offered by the differential in gel electrophoresis technology (DIGE), in which the gel-to-gel variance is solved for comparative proteomics. In fact, in this technology, up to three samples can be differentially labeled and run on the same gel, increasing confidence in the detection and quantification of differences in protein abundance, taking advantage of the introduction of an internal standard that suppresses inter-gel variability [40]. There are only a few examples of the application of this technology to the study of food products, and these are related to the characterization of breed muscle proteome profiles [41], to investigate changes between GMOs and their counterpart non-GMOs [42], and very recently to the study of shelf-life storage process in fresh-cut and ready-to-eat vegetables [43].

Fish and prawn species authentication case.

Marine species in seafood products are often subjected to authentication not just for commercial fraud due to replacing quality materials with low-level ones, but also because they contain allergenic or toxic compounds dangerous for the health of consumers. Therefore, European legislation recommends seafood products labeling (Regulation (EU) No 1379/2013 of the European Parliament and of the Council of 11 December 2013 on the common organisation of the markets in fishery and aquaculture products).

2-DE proteomics approach thanks to sensitivity and possibility to be applied at a large-scale demonstrated to be a suitable analytical method to distinguish seafood species and to quantify their levels in seafood products.

Different 2-DE protein profiles of water-soluble proteins have been used to discriminate very closely related fish species [44]. In particular, studies based on 2-DE analysis revealed proteins such as triose phosphate isomerase isoforms, pyruvate kinase, troponin T, and beta-enolase as specific markers for distinguishing various species of tuna as Thunnus thynnus, Thunnus alalunga, Thunnus albacares, and Thunnus obesus [45]. A 2-DE approach has also revealed a different electrophoretic mobility of several spots together with a qualitative presence in farmed cod samples allowing to differentiate wild cods from farmed ones [46]. Moreover, few studies have been focused on crustaceans, known to be highly allergenic, to highlight differences among the closely related group of Decapoda shrimps and prawns. A proteomic gel-based approach successfully revealed different protein patterns in the most commercially relevant shrimp species. 2-DE profiles showed that the sarcoplasmic protein arginine kinase (AK) isoforms were differently modulated in six different species, proposing it as a biomarker for discrimination of Decapoda species also in mixed food products [47]. Moreover, 2DE represents also a useful method for food security purposes since it allows to select and quantify the levels of this protein, known to be highly allergenic.

3.2 Gel-free approaches

Despite the robustness of 2-DE techniques, gel-based proteomics suffers from intrinsic limitations, that prevent the separation of highly hydrophobic, extreme isoelectric point, or high MW proteins. Therefore, the scientific community has oriented, in recent years, in favour of complementary methods, globally known as mass spectrometry (MS)-based proteomics. This type of approach is rapidly emerging as a pivotal proteomic technology for the determination of food quality, authenticity, functionality, and safety.

It is characterized by a very large variety of analytical strategies and instrumentations whose detailed description is beyond the scope of this review. Nevertheless, all the different MS-based proteomic approaches share a common workflow, made up of three fundamental stages: 1) isolation and (pre)fractionation of protein sample; 2) qualitative and quantitative analysis by MS or MS/MS and 3) and assignment of MS or MS/MS spectra to peptides and proteins through database searching [48] (Figure 3).

Basically, two different workflows have been currently developed: bottom-up and top-down approaches. In the first, usually referred as shotgun proteomics, protein sample at stage 1 is enzymatically digested and the resulting complex peptide mixture is subjected to fractionation and analyses. Conversely, top-down proteomics is based on pre-fractionation, injection, and analyses of intact proteins. With respect to quantitative analysis, MS-based proteomics

offers at least three different approaches: label-based, label-free and targeted quantitation methods. We refer the readers to specific reviews for further information [49].

Targeted analyses offer the possibility to monitor and quantify specific proteins of interest (e.g. biomarkers). Multiple reaction monitoring (MRM) is becoming the central platform in for targeted analysis of protein/peptide abundances in complex matrices, food included. Depending on the type of instrumentation used, MRM assays can perform multiplexed analyses of hundreds of peptides in a single run [50].

The Milk case

Milk and dairy products have been extensively investigated by using proteomics [51]. Milk is characterized by a wide dynamic range, displaying high abundance proteins (e.g. caseins) and medium to low abundance whey proteins (e.g. ß-lactoglobulin, lactoferrin, immunoglobulins, glycoproteins, peptide hormones, and enzymes) [52]. First studies were addressed to the characterization of breast milk for its importance in newborn nutrition. The "classical" 2DE-MS proteomic approach led to the identification of 107 protein spots, corresponding to 39 gene products, in milk fat globule membrane proteins [53]. More recent works based on gel-free shotgun deeply penetrated into human milk proteome, identifying 268 gene products by using cutting-edge MS platform [54]. The evolution of high-throughput MS-technologies has paved the way to a more detailed characterization of breast milk, cow's milk, and dairy products. Milk transformation requires heating processes to guarantee its microbiological safety and a longer shelf-life. However, thermal treatments result in chemical modifications of milk proteins, affecting their nutritional, nutraceutical, biological and toxicological properties. Shotgun proteomic approach has been applied for protein structural analysis of glycation and glycoxidation in raw, pasteurized, UHT and powdered infant formula milk samples, identifying several protein targets in diverse heated samples [55]. MS-based technologies implementing MRM (Multiple Reaction Monitoring) scan function offer the possibility to detect and quantify specific biomarkers in complex food matrices. This strategy has been successfully applied for the detection of adulteration in buffalo mozzarella, by monitoring and quantifying the phosphorylated β-casein f33-48 peptide, identified as a specific proteotypic marker [56].

In conclusion, the rapid advances in proteomics, mainly represented by the use of gel-free approaches alongside with the more traditional gel-based methodologies, are providing valuable data such as quality/quantitative protein biomarkers that, in some cases, already found an application in the validation of industrial processes of food products and in the assessment of food quality, safety and traceability.

4. Transcriptomics

The study of a total set of transcripts in a given organism (namely transcriptome), at any given time and under any condition, allows to obtain meaningful insights into the functional elements of a genome and to elucidate molecular mechanisms underlying complex biological processes.

Comparative analysis of the transcriptome composition is a powerful tool to identify transient alterations in gene expression in response to genetic and/or environmental cues and in response to the general metabolic state of a given cell or tissue. This approach has been widely used to improve quality and quantity of food crops, as well as to characterize quality and safety of food (i.e. detection of transcripts involved in the accumulation of contaminants, bioactive molecules, nutrients, health effect molecules, etc.) [11].

High throughput transcriptomics became first possible with the development of the microarrays technique through the interrogation of thousands of gene targets by hybridization of RNA samples to oligonucleotide probes laying on miniaturized devices (Figure 4) [57].

In food crops, microarray-based approaches have been particularly useful to compare fluctuations in gene expression profiles in response to a wide range of conditions, like drought, high-salinity, cold, heat and disease [58]. The major drawback is that microarrays can detect only known gene sequences so they can't be used for the discovery of alterations of unknown genes. However, in the past few years, transcriptomics has expanded dramatically because of the advent of next generation sequencing (NGS) and genome-wide RNA sequencing (RNA-seq) (Figure 4) [59]. This latter is a more versatile technology to detect alterations of the transcriptome and is progressively overtaking the microarray-based approaches. It can easily provide, in fact, high-resolution gene expression, detection of lower abundant transcripts, point mutations, alternative splicing as well as new coding and noncoding RNA transcripts, including small regulatory RNAs (sRNAs) and antisense transcripts [60].

Nowadays, the massive sequencing data available on the genome structure of thousands of species has allowed performing transcriptomic assays based on genome-wide microarrays and RNAseq on many crop and animal species to investigate gene functions involved in the accumulation of compounds of interest for human health and for the overall food quality. The intrinsic nature of the RNA-based investigations requires fresh food substrates with minimal processing. RNA molecules are very delicate and undergo a quick degradation as cell structures lose their metabolic activity and are dismantled during the preparation of certain food products which require high temperatures and complex manipulation of the basic ingredients. In such foods, transcriptomic investigations become in fact more and more challenging and are conveniently replaced by other biochemical assays to detect proteins or metabolites.

Nevertheless, transcriptomics of fresh unprocessed food products has proven to be very effective as a diagnostic tool to infer about many attributes of foods through detection of altered gene expression. In this context, an example of a recent transcriptomic study elucidate biosynthetic processes underlying the regulation of bitter taste in chicory, which is a crucial attribute of quality in terms of the product acceptability [61]. It's known that sesquiterpene lactones (STLs), secondary metabolites typical of Asteraceae spp., confer the bitterness of chicory, in addition to other nutritional, allergenic and healthy properties. Analysis of differentially expressed genes in two stem-chicory "Catalogna" landraces, characterized by different bitterness scores, identified several STL genes strictly associated to bitterness which promise to be useful markers for food quality.

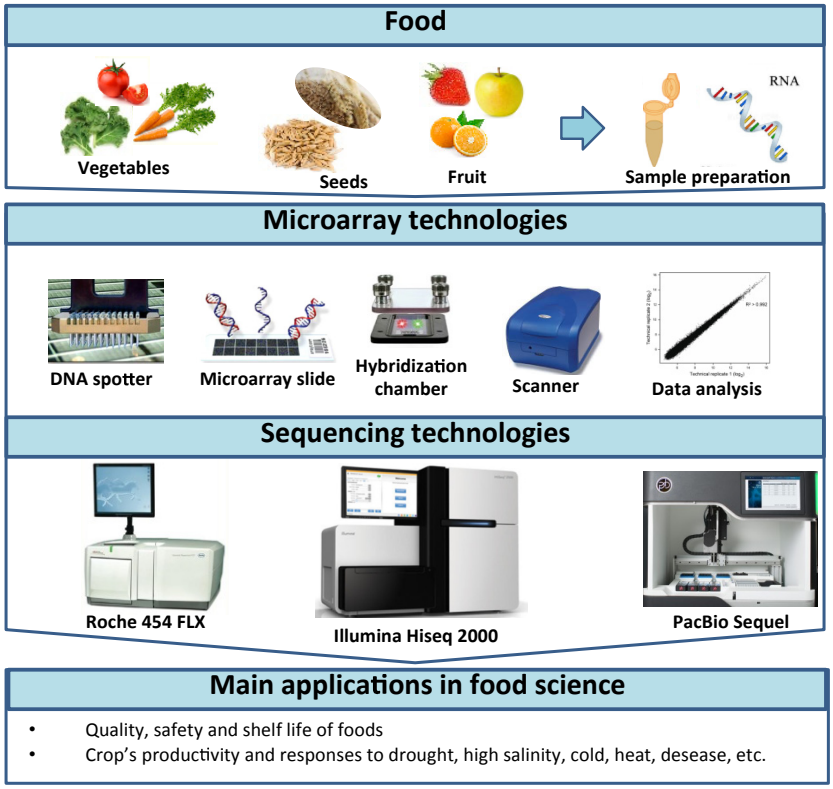


Fig. 4 Overview of food transcriptomics.

Gene expression analysis is also useful to enhance food quality and improve the shelf life of products by investigating molecular mechanisms which take place during storage and post-harvest treatments. Mellidou and colleagues explored changes of the apple transcriptome associated with a flesh browning disorder related to controlled atmosphere storage [62]. The identified candidate genes associated with internal browning in a tissue-specific manner represent markers of the browning incidence in apples during storage in the controlled atmosphere and could be useful to improve the postharvest life of apple, as well [62].

A further application of transcriptomics is to detect food contaminants by fingerprinting methodologies [12]. Living cells display wide adaptive plasticity by responding with characteristic expression changes to the toxic agents possibly present in foods. Two human cell lines have been used to explore the impact of microarray-based transcriptomic analyses for detection of different contaminants. Interestingly, both cell lines yielded characteristic expression profiles following the exposure to chemicals that are often found in food products. In this context, the transcriptomics approach can expand the range of contaminants that can be detected in a single experiment and increase the specificity of the cellular response [12].

Transcriptomic techniques have demonstrated their impressive analytical potential for gene expression studies in the context of food science. However, the applicability of RNA-based assays in food science is still in its infancy and it is not fully exploited yet.

Though RNA-Seq has quickly superseded microarrays in many gene expression studies, it still remains evolving and several technical and bioinformatics challenges need to be overcome to realize the full potential of this technique in food science. Given the evolution path of RNA-Seq technology, high-end instruments with higher sequencing throughput able to provide longer and accurate reads can be expected in the very near future. For instance, a new generation of sequencers, based on single- molecule and single- cell sequencing, is rapidly emerging [63]. In addition to their capability for sequencing RNA directly, novel technologies can also sequence molecules in real-time, decreasing the time of analysis and allowing higher sensitivity. The improved analytical performances made available

by the new sequencing technologies can provide more detailed information about the transcriptional regulation in real samples boosting the applicability of transcriptomics in food science.

5. Metabolomics

The study of the entire metabolite composition of a living cell, organism or a particular system has been defined as metabolome, and metabolomics allows the determination of the metabolic composition of different matrices. Since the metabolome represents the final biochemical results of gene expression and environmental conditions, metabolomic approaches have emerged as reliable and powerful tools able to extend the knowledge on food biochemical composition and to investigate the quality and safety of different food matrices [64]. Metabolomics basically comprises targeted and untargeted approaches [65]. While targeted metabolomics aims to selectively profile several metabolites, and usually requires a specific extraction of metabolites, the untargeted approach is used to extrapolate significant differences in a comparative manner, allowing to apply a metabolic fingerprinting or to identify new compounds as biomarkers for food safety and quality [66].

Like other "omic" disciplines, metabolomics involves instrumental analysis, which is optimized to obtain the most comprehensive metabolic data set, and data analysis workflow, which consists of the bioinformatics/statistic tools used to process high-throughput data sets. Key steps for obtaining such metabolic data set are i) extraction, ii) separation, and iii) detection of metabolites (Figure 5).

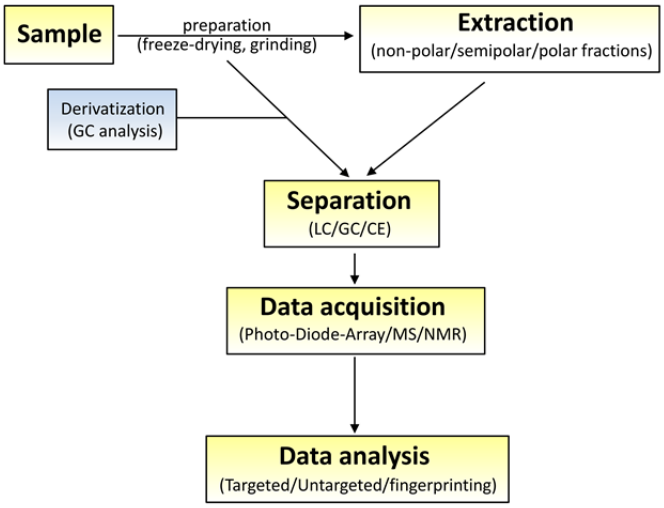


Fig. 5 Pipeline for food metabolomics.

Different protocols have been established for the extraction of specific classes of metabolites or for global profile studies [66]. In the targeted approach metabolites extraction often includes sample clean-up step using Solid Phase Extraction (SPE) or Solid Phase Micro-extraction (SPME), while to obtain a global metabolite profile different extraction protocols are used in order to increase the metabolites diversity. The separation step is in general accomplished by liquid chromatography (LC), mainly with its high performance (HPLC), ultra-performance (UPLC) or ultra-high performance (UHPLC) forms, gas chromatography (GC) and capillary electrophoresis (CE). However, in some cases, direct infusion (DI) is used to obtain a metabolic profile. Numerous techniques have been developed for metabolites detection, such as Fourier transform infra-red spectroscopy (FTIR) [15], nuclear magnetic resonance (NMR) [16] and mass spectrometry (MS) [17] although in food metabolomics MS and NMR have been used the most [65]. While NMR gives high-resolution spectra, allowing to identify the exact structure of metabolites, in MS the mass analyzers used ranged from high resolution MS (HRMS) [17], such as Fourier transform ion cyclotron resonance (FTICR), Orbitrap and Time of Flight (ToF), to low-resolution MS like quadrupole (Q), ion-trap (IT), ion-mobility spectrometry (IMS) and hybrid systems. Clearly, HRMS is preferred because of its high mass accuracy and sensitivity [17]. The acquired data are then analyzed using different statistical and chemometric approaches. Many metabolite databases have been developed, helping the identification of unknown metabolites present in food matrices. Due to the advancements in analytical instruments in the last decade, it is now possible to analyze thousands of metabolites from a food sample in a single analysis. The sample preparation and data handling processes have also been improved tremendously, making it easier for scientists to analyze samples in a short time.

In the frame of food quality, one of the major challenges of metabolomics is to develop fast, reproducible and cost-effective analytical methods able to assess food quality, with the ultimate purpose to protect and improve the health of consumers, satisfying their expectations. Although the quality of raw materials is basically due to intrinsic traits of the

sample, the final products of food processing derive from raw ingredients combined and transformed to produce marketable products, thus final food-quality is given by multiple processing factors, as well as by storage and packaging conditions. A large number of MS-based and HRMS-based approaches have been applied in order to improve food quality, developing robust and fast methods able to analyse the wide range for nowadays known health-related metabolites, such as phytochemicals (carotenoids, polyphenols, see for example [67]), vitamins (provitamin A and vitamin C) [68] fatty acids (omega-3 and omega-6 FA) [69] and minerals (calcium, potassium and magnesium) [70]. Recent MS-based approaches have been used, for instance, to simultaneously quantify several abundant bioactive compounds such as carotenoids, tocopherols and free and esterified sterols, in important food product such as canola, olive, and sunflower oils, by using a target HPLC-DAD-MS/MS strategy, a simple extraction procedure and 30 minutes total runtime [71].

Since new MS technologies are able to produce large dataset with thousands of potentially quality-related compounds, metabolomic approaches have been used in combination with multivariate analysis, such as PCA (Principal Component Analysis), in order to extrapolate significant information, as has been described, for instance, for selected specific metabolite patterns discriminating fruit from several commercially grown cultivars, demonstrating that specific metabolites correlate directly with quality traits [72]. Since flavour is one of the most important criteria influencing consumer acceptance and quality of foods, an increasing number of GC-MS based approaches have been extended to discover metabolites that significantly contribute to these traits. GC-MS based approach has been recently used to identify 33 compounds correlating with consumer liking, and 37 that significantly correlated with flavor intensity. These compound have been further used to design a genome-wide association study (GWAS), identifying candidate loci capable of altering 15 of the chemicals contributing to consumer liking and an additional 6 chemicals that contribute to overall flavour intensity of tomatoes [73].

In addition, metabolomics represents a powerful tool for the determination of food contaminants like toxins (mycotoxins, mainly found in cereals, and algal toxins that contaminate marine-related foods) [74], chemicals (including pesticides, pollutants, antibiotics and growth-promoting agents) [75], spoilage microorganism (*Pseudomonas spp*., *Acinetobacter spp*., *Botrytis spp*.) and pathogens (*Escherichia coli* strains, *Salmonella spp*., *Shigella spp*., *Listeria monocytogenes*, *Campylobacter jejuni* and viruses, such as norovirus and rotavirus) [76] or genetic modified (GM) ingredients [77].

Both targeted and non-targeted metabolomics have been used for the detection and identification of such contaminants. While targeted analysis by using NMR or MS are suitable for the study of already-known contaminants, the untargeted approach is indicated to identify novel compounds that can be used as biomarkers for the identification of illegal practices in food production and to monitor the integrity and safety of food products.

The method of choice for toxins detection is represented by LC-HRMS or LC-MS/MS. Simultaneous detection of several toxins has been successfully reported by the use of high-resolution analyzer [78]. LC-HRMS protocols were also established in the case of chemicals identification such as pesticides, antibiotics and growth-promoting agents [75], and comparative metabolic profiles of GM and non-GM crops have been performed using targeted and untargeted LC-HRMS and NMR [80]. Metabolic fingerprinting using GC-MS have been efficiently established for the determination of spoilage or pathogenic microorganisms that produce volatile organic compounds (VOC) [80] [81]. Other primary metabolites such as sugars and amino acids were characterized as biomarkers for early food contamination by pathogens [81]. GC-MS also remain the best technique for the determination of environmental pollutants in food such as polychlorinated biphenyls, polycyclic aromatic hydrocarbons or polybrominated diphenyl ethers [82].

6. The need for data integration: system biology and foodomics

The development of high-throughput technologies has led to the generation of large “omics” dataset that allowed the identification of novel biomarkers for food safety and quality. However, considering the dynamics of biological systems, multi-level integration studies can result particularly useful in this context. Integration of genomics, transcriptomics, proteomics and especially metabolomics, to study the interactions among molecular components and their changes induced by perturbations in a biological system is an emerging discipline named systems biology [83]. Basically, two approaches, the data-driven and the model-driven, are used to study biological systems [83] [84]. The first approach is useful to identify novel components and their interactions from large-scale omics datasets in order to define new metabolic functions. In general, molecular interactions are analyzed by correlation networks that can be visualized as graphs in which the components are described by nodes and the interactions are shown as edges [85]. The model-driven approach can be then used to interpret the behavior of the system. In general, a mathematical model of the biological system is constructed and matched against global observations in an iterative manner, in order to obtain a model that reflects biological reality [85].

The development of systems biology is beneficial to the study of the impact of food compositions and ingredients on human health [20]. The traditional food research is moving from classical methodological methods to new analytical approaches aiming at integrating biological data with bio-informatics tools. The new omics technologies combined with system biology can lead food research into a new era, the foodomics, which was defined in 2009 as a discipline that

studies the food and nutrition domains through the application and integration of advanced omics technologies to improve consumer's well-being, health, and knowledge [86]. Currently, in the so-called globalization, in which the movement of food and related raw-materials worldwide requires the ensuring of safety, quality, and traceability of products, the development, validation, and implementation of rapid, sensitive, and accurate methods for assessment of food safety are needed [87]. Foodomics involves the use of genomics, proteomics, transcriptomics, and metabolomics, including nutrigenomics, for compound profiling, authenticity, and/or biomarker detection related to food quality and safety [2]. It has already been applied in different fields of science and food technology and nutrition; i.e., evaluation of food safety and quality, the study of the effects on human health of different bioactive food components, the individuation and quantification of dietary biomarkers related to different health conditions, or the assessment of biological responses to different nutritional patterns. An especially complex challenge in the combination of Foodomics and system biology is the possibility of connecting food components, foods, diet, the individual, including food impact on health and illness, by considering the food domain as a whole with the nutrition domain. Only in this way, it will possible to account for food products tailored to promote human health and well-being.

References

[1] Bock AK, Maragkoudakis P, Wollgast J, Caldeira S, Czimbalmos A, Rzychon M, et al. Tomorrow's healthy society - research priorities for foods and diets. Final Report. JRC Foresight Study; 2014. Publications Office of the European Union. Report No.:10.2788/14108.

[2] Josić D, Peršurić Ž, Rešetar D, Martinović T, Saftić L, Kraljević Pavelić, S. Chapter Six - Use of foodomics for control of food processing and assessing of food safety. In: Fidel Toldrá, Editor(s). Advances in Food and Nutrition Research. Academic Press; 2017. Vol 81, p. 187–229.

[3] Schwab K. Shaping the future of global food systems: A scenarios analysis.World Economic Forum Report; 2017 Jan.

[4] Bergholz TM, Moreno Switt AI, Wiedmann M. Omics approaches in food safety: fulfilling the promise? Trends Microbiol. 2014; 22(5):275–81.

[5] Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, et al. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature. 2000; 403(6770):665–68.

[6] Bell RL, Jarvis KG, Ottesen AR, McFarland MA, Brown EW. Recent and emerging innovations in *Salmonella* detection: a food and environmental perspective. Microb Biotechnol. 2016; 9(3):279–92.

[7] Roberts MA, Mutch DM,German JB. Genomics: food and nutrition. Curr Opin Biotechnol. 2001; (12):516-22.

[8] Leff JV, Fierer N. Bacterial communities associated with the surfaces of fresh fruits and vegetables. PLoS One. 2013; 8(3):e59310.

[9] Ercolini D. High-throughput sequencing and metagenomics: moving forward in the culture-independent analysis of food microbial ecology. Appl Environ Microbiol. 2013; 79(103):148–55.

[10] Mayo B, Rachid CT, Alegría Á, Leite AM, Peixoto RS, Delgado S. Impact of next generation sequencing techniques in food microbiology. Curr Genomics. 2014; 15(4):293–309.

[11] Valdés A, Ibáñez C, Simó C, García-Cañas V. Recent transcriptomics advances and emerging applications in food science. TrAC Trends Anal Chem. 2013; 52:142–54.

[12] Lancova K, Dip R, Antignac JP, Le Bizec B, Elliott CT, Naegeli H. Detection of hazardous food contaminants by transcriptomics fingerprinting. TrAC Trends Anal Chem. 2011; 30(2):181–91.

[13] Gašo-Sokač D, Kovač S, Josić D. Application of proteomics in food technology and food biotechnology: Process development, quality control and product safety. Food Technol Biotechnol. 2010; 48(3):284–95.

[14] Piras C, Roncada P, Rodrigues PM, Bonizzi L, Soggiu A. Proteomics in food: Quality, safety, microbes, and allergens. Proteomics. 2016; 16(5):799–815.

[15] Baker MJ, Trevisan J, Bassan P, Bhargava R, Butler HJ, Dorling KM, et al. Using Fourier transform IR spectroscopy to analyze biological materials. Nat Protoc. 2014; 9(8):1771–91.

[16] Markley JL, Brϋschweiler R, Edison AS, Eghbalnia HR, Powers R, Raftery D, Wishart DS. The future of NMR-based metabolomics. Curr Opin Biotechnol. 2017; 43:34–40.

[17] Rathahao-Paris E, Alves S, Junot C, Tabet JC. High resolution mass spectrometry for structural identification of metabolites in metabolomics. Metabolomics. 2016; 12(1):10.

[18] Kitano H. Systems biology: A brief overview. Science. 2002; 295(5560):1662–64.

[19] Orešič M. Systems Biology in Food and Nutrition Research. In: Foodomics. John Wiley & Sons Inc. 2013; pp. 539–50.

[20] Josić D, Giacometti J. Foodomics-use of integrated omics in nutrition, food technology and biotechnology. J Data Mining Genomics Proteomics. 2013; 4:e106.

[21] Ferri E, Galimberti A, Casiraghi M, Airoldi C, Ciaramelli C, Palmioli A, et al. Towards a universal approach based on omics technologies for the quality control of food. Biomed Res Int. 2015; 365794.

[22] Begley M, Hill C. Food safety: what can we learn from genomics? Annu Rev Food Sci Technol. 2010; 1(1): 341–61.

[23] Chen Y, Burall LS, Luo Y, Timme R, Melka D, Muruvanda T, et al. *Listeria monocytogenes* in stone fruits linked to a multistate outbreak: Enumeration of cells and whole-genome sequencing. Appl Environ Microbiol. 2016; 82(24): 7030–40.

[24] Nieuwenhuijse DF, Koopmans MPG. Metagenomic sequencing for surveillance of food- and waterborne viral diseases. Front Microbiol. 2017;8: 230.

[25] Ceuppens S, Dan Li, Uyttendaele M, Renault P, Ross P, Van Ranst M, Cocolin L, Donaghy J. Molecular methods in food safety microbiology: Interpretation and implications of nucleic acid detection. Comprehensive Review in Food Sci Food Saf. 2014;13(4):551–77.

[26] Shin HH, Hwang BH, Cha HJ. Multiplex 16S rRNA-derived geno-biochip for detection of 16 bacterial pathogens from contaminated foods. Biotechnol J. 2016;11(11):1405–14.
[27] ISO. 22119: 2011. Microbiology of food and animal feeding stuffs - Real time polymerase chain reaction (PCR) for the detection of food-borne pathogens - general requirements and definitions.
[28] ISO. 22118: 2011. Microbiology of food and animal feeding stuffs - Real time polymerase chain reaction (PCR) for the detection of food-borne pathogens - performance characteristics.
[29] Walsh AM, Crispie F, Marcus J C, Cotter PD. Translating omics to food microbiology. Annu Rev Food Sci Technol. 2017;(8):113–34.
[30] Ronholm J, Nasheri N, Petronella N, Pagotto F. Navigating microbiological food safety in the era of whole-genome sequencing. Clin Microbiol Rev. 2016;29(4):837–57.
[31] Giello M, La Storia A, Masucci F, Di Francia A, Ercolini D, Villani F. Dynamics of bacterial communities during manufacture and ripening of traditional Caciocavallo of Castelfranco cheese in relation to cows' feeding. Food Microbiol. 2017;63:170–77.
[32] Cao J, Yang J, Hou Q, Xu H, Zheng Y, Zhang H, Zhang L. Assessment of bacterial profiles in aged, home-made Sichuan paocai brine with varying titratable acidity by PacBio SMRT sequencing technology. Food Control. 2017;78:14–23.
[33] Muñoz-Colmenero M, Martínez JL, Roca A, Garcia-Vazquez E. NGS tools for traceability in candies as high processed food products: Ion Torrent PGM versus conventional PCR-cloning. Food Chem. 2017;214:631–36.
[34] DeSantis TZ, Hugenholtz P, Larsen N, Rojas M,. Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006;72(7):5069–72.
[35] Cole JR, Wang Q, Fish A, Chai B, McGarrell DM, Sun Y, et al. Ribosomal Database Project: Data and tools for high throughput rRNA analysis. Nucleic Acids Res. 2014;42(DI): D633-42.
[36] Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 2013;41(DI):D590-96.
[37] D'Alessandro AD, Zolla L. Food Safety and Quality Control : Food safety and quality control: Hints from Proteomics. Food Technol Biotechnol. 2012;50(3):275–85.
[38] Zamboni A, Di Carli M, Guzzo F, Stocchero M, Zenoni S, Ferrarini A, et al. Identification of putative stage-specific grapevine berry biomarkers and omics data integration into networks..Plant Physiol. 2009;154(3):1439–59.
[39] Pandey A, Mann M. Proteomics to study genes and genomes. Nature. 2000;405:837–46.
[40] Minden JS, Dowd SR, Meyer HE, Stühler K. Difference gel electrophoresis. Electrophoresis. 2009;30: S156-S161.
[41] Hollung K, Grove H, Faergestad EM, Sidhu MS, Berg P. Comparison of muscle proteome profiles in pure breeds of Norwegian Landrace and Duroc at three different ages. Meat Sci. 2009;81(3):487–92.
[42] Di Carli M, Villani ME, Renzone G, Nardi L, Pasquo A, Franconi R, et al. Leaf proteome analysis of transgenic plants expressing antiviral antibodies. J Proteome Res. 2009;8(2):838–48.
[43] Di Carli M, De Rossi P, Paganin P, Del Fiore A, Lecce F, Capodicasa C, et al. Bacterial community and proteome analysis of fresh-cut lettuce as affected by packaging. FEMS Microbiol Lett. 2016;363(1):fnv209.
[44] Piñeiro C, Vázquez J, Marina AI, Barros-Velézquez J, Gallardo JM. Characterization and partial sequencing of species-specific sarcoplasmic polypeptides from commercial hake species by mass spectrometry following two-dimensional electrophoresis. Electrophoresis. 2001;22(8):1545–52.
[45] Pepe ML, Ceruso T, Carpentieri M, Ventrone A, Amoresano I, Anastasio A, Cortesi A. Differentiation of four Tuna species by two-dimensional electrophoresis and mass spectrometric analysis. In: Heazlewood JH and Petzold CJ, editors; Proteomic Applications in Biology; 2012. p. 191-208.
[46] Martinez I, Šližytė R, Daukšas E. High resolution two-dimensional electrophoresis as a tool to differentiate wild from farmed cod (*Gadus morhua*) and to assess the protein composition of klipfish. Food Chem. 2007; 102(2): 504–10.
[47] Ortea I, Canas B, Calo-Mata P, Barros-Velázquez J, Gallardo M. Arginine kinase peptide mass fingerprinting as a proteomic approach for species identification and taxonomic analysis of commercially relevant shrimp species. J Agric Food Chem. 2009;57(13):5665–72.
[48] Picariello G, Mamone G, Addeo F, Ferranti P. Novel mass spectrometry-based applications of the 'Omic' sciences in food technology and biotechnology. Food Technol Biotechnol. 2012;50 (3):286–305.
[49] Mena MdC, Albar JP. Next generation instruments and methods for proteomics. In: Foodomics: Advanced mass spectrometry in modern food science and nutrition, 2013. Cifuentes A, editor. John Wiley & Sons, Inc., Hoboken, NJ, USA.
[50] Liebler DC, Zimmerman LJ. Targeted quantitation of proteins by mass spectrometry. Biochemistry. 2013; 52 (22):3797–806.
[51] Arena SA, Renzone G, D'Ambrosio C, Salzano AM. Dairy products and the Maillard reaction: A promising future for extensive food characterization by integrated proteomics studies. Food Chem. 2017;219:477–89.
[52] Vincent D, Ezernieks V, Elkins A, Nguyen N, Moate PJ, Cocks BG, Rochfort S. Milk bottom-up proteomics: Method optimization. Front Genet. 2016;6:360.
[53] Cavaletto M, Giuffrida MG, Conti A. The proteomic approach to analysis of human milk fat globule membrane. Clin Chim Acta. 2004; 347(1–2):41–48.
[54] Picariello G, Mamone G. Gel-free shotgun proteomic analysis of human milk.. J Chromatogr. 2012;1227:219-33.
[55] Renzone G, Arena SA, Scaloni A. Proteomic characterization of intermediate and advanced glycation end-products in commercial milk samples. J. Proteomics. 2015;117:12–23.
[56] Russo R, Severino V, Mendez A, Lliberia J, Parente A, Chambery A. Detection of buffalo mozzarella adulteration by an ultra-high performance liquid chromatography tandem mass spectrometry methodology. J Mass Spectrom. 2012;47(11):1407–14.
[57] Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science.1995;270(5235):467–70.
[58] Vij S, Tyagi AK. Emerging trends in the functional genomics of the abiotic stress response in crop plants. Plant Biotechnol J. 2007;5(3):361–80.
[59] Metzker ML. Sequencing technologies - the next generation. Nat Rev Genet. 2010;11(1):31–46.
[60] Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet. 2009;10 (1):57–63.

[61] Testone G, Mele G, Di Giacomo E, Gonnella M, Renna M, Tenore GC, et al. Insights into the sesquiterpenoid pathway by metabolic profiling and de novo transcriptome assembly of stem-chicory (Cichorium intybus Cultigroup 'Catalogna'). Front Plant Sci. 2016;(7):1676.
[62] Mellidou I, Buts K, Hatoum D, Ho QT, Johnston JW, Watkins CB, et al. Transcriptomic events associated with internal browning of apple during postharvest storage. BMC Plant Biol. 2014;14:328.
[63] Gawad C, Koh W, Quake SR. Single-cell genome sequencing: current state of the science. Nat Rev Genet. 2016;17(3):175–88.
[64] Pinu FR. Metabolomics: Applications to food safety and quality research. In: Beale DJ, Kouremenos KA, Palombo EA, editors. Microbial metabolomics: Applications in clinical, environmental, and industrial microbiology, Cham: Springer International Publishing; 2016. p. 225–59.
[65] Gorrochategui E, Jaumot J, Lacorte S, Tauler R. Data analysis strategies for targeted and untargeted LC-MS metabolomic studies: Overview and workflow. TrAC Trends Anal Chem. 2016;82:425–42.
[66] Cevallos-cevallos JM, Etxeberria E, Danyluk MD, Rodrick GE. Metabolomic analysis in food science: a review. Trends Food Sci Technol. 2009;20(11–12):557–66.
[67] Tsao R. Chemistry and biochemistry of dietary polyphenols. Nutrients. 2010; 2(12):1231–46.
[68] Combs GF, McClung JP. The Vitamins, Fifth Edition. Fundamental Aspects in Nutrition and Health. Academic Press; 2017.
[69] de Lorgeril M, Salen P. New insights into the health effects of dietary saturated and omega-6 and omega-3 polyunsaturated fatty acids. BMC Med. 2012;10:50.
[70] McLean E, Cogswell M, Egli I, Wojdyla D, de Benoist B. Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993–2005. Public Health Nutr. 2009;12(4):444-54.
[71] Flakelar CL, Prenzler PD, Luckett DJ, Howitt JA, Doran G. A rapid method for the simultaneous quantification of the major tocopherols, carotenoids, free and esterified sterols in canola (*Brassica napus*) oil using normal phase liquid chromatography. Food Chem. 2016; 214:147–55.
[72] Cuthbertson D, Andrews PK, Reganold JP, Davies NM, Lange BM. Utility of metabolomics toward assessing the metabolic basis of quality traits in apple fruit with an emphasis on antioxidants. J Agric Food Chem. 2012;60(35):8552–60.
[73] Tieman D, Zhu G, Resende MFR, Lin T, Nguyen C, Bies D, Rambla JL, Beltran KSO, et al. A chemical genetic roadmap to improved tomato flavor. Science. 2017;355(6323):391-94.
[74] Giacometti J, Tomljanović AB, Josić D. Application of proteomics and metabolomics for investigation of food toxins. Food Res Int. 2013;54(1):1042–51.
[75] Hird S J, Lau Y B P, Schuhmacher R, Krska R. Liquid chromatography-mass spectrometry for the determination of chemical contaminants in food. TrAC Trends Anal Chem. 2014;59:59–72.
[76] Pinu F R. Early detection of food pathogens and food spoilage microorganisms: Application of metabolomics. Trends Food Sci Technol. 2016;54:213–15.
[77] Simó C, Ibáñez C, Valdés A, Cifuentes A, García-Cañas V. Metabolomics of genetically modified crops. Int J Mol Scie. 2014;15(10):18941–66.
[78] Lattanzio VM, Gatta S, Godula M, Visconti A. Quantitative analysis of mycotoxins in cereal foods by collision cell fragmentation-high-resolution mass spectrometry: performance and comparison with triple-stage quadrupole detection. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2011;28(10):1424–37.
[79] Hrbek V, Krtkova V, J. Rubert J, Chmelarova H, Demnerova K, Ovesna J, Hajslova J. Metabolomic strategies based on high-resolution mass spectrometry as a tool for recognition of GMO (MON 89788 Variety) and non-GMO soybean: a critical assessment of two complementary methods. Food Anal Methods. 2017;1–15.
[80] Wang Y, Li Y, Yang J, Ruan J, Sun C. Microbial volatile organic compounds and their application in microorganism identification in foodstuff. TrAC Trends Anal Chem. 2016;78: 1–16.
[81] Cevallos-Cevallos JM, Danyluk MD, Reyes-De-Corcuera JI. GC-MS based metabolomics for rapid simultaneous detection of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Salmonella* Muenchen, and *Salmonella* Hartford in ground beef and chicken. J Food Sci. 2011;76 (4):M238–46.
[82] Tsipi D, Botitsi H, Economou A. Mass spectrometry for the analysis of pesticide residues and their metabolites. 2015.Wiley Series on Mass Spectrometry.
[83] Fukushima A, Kusano M, Redestig H, Arita M, Saito K. Integrated omics approaches in plant systems biology. Curr Opin Chem Biol.2009;13(5–6):532–38.
[84] Ideker T, Galitski T, Hood L. A new approach to decoding life: Systems Biology. Annu Rev Genomics Hum Genet. 2001;2:343–72.
[85] Saito K, Matsuda F. Metabolomics for Functional Genomics, Systems Biology, and Biotechnology. Annu Rev Plant Biol 2010; 61(1):463–89.
[86] Garcia-Cañas V, Simó C, Herrero M, Ibáñez E, Cifuentes A. Present and future challenges in food analysis: Foodomics. Anal Chem. 2012; 84(23):10150–59.
[87] Iannetta M, Matranga G, Zoani C, Canese S, Daroda L, Vitali F, Zappa G. Innovation in logistics and in the supply chain integrated approach. In: CIHEAM, editor. Logistics and agro-food trade and challenge for the Mediterranean. Mediterra 2014. Chapter 28. Publisher: SciencesPo. LesPresses, p.463-476.

Lipid Peroxidation and Its Antimicrobial Effect in Foods

A. Kocabaş[1] and **S. Gökmen**[2]

[1] Department of Biology, Science Faculty, Karamanoğlu Mehmetbey University, Karaman, Turkey
[2] Department of Food Processing, Vocational School of Technical Sciences, Karamanoglu Mehmetbey University, Karaman, Turkey

Rancidity is lipid peroxidation process and an undesirable phenomenon occurred in foods having unsaturated fats and oils. It resulted in food deterioration. Therefore, there is a limitation of lipid peroxidation for food quality control as a value of TBA. Malonaldehyde (MDA) is the last product of lipid autoxidation and Thiobarbituric Acid (TBA) is the most widely used assay to detect lipid peroxidation in foods. Although rancidity is inevitable process, it can be controlled by some additives or processes. In this research, it was found that increasing lipid peroxidation level in Manti is inversely proportional to the microbiological load.

Keywords: Lipid Peroxidation, Antimicrobial Effect, TBA

1. Introduction

The oxidation of unsaturated fatty acids because of physical factors (temperature, pH) or biological reasons such as enzymatic degradation in foods called rancidity. Rancidity results in the formation of sensory and biological undesirable products, the result of which is a sign of deterioration in foods. After first step of autoxidation, lipid peroxidation shows geometric increase in foods. Keeping it under control therefore is important for the quality of food.

Lipid peroxidation is a chain reaction initiated by the formation of hydroperoxides. It occurs in two different ways. 1. Autoxidation a) free radical mechanism b) photo-oxidation and 2) mechanism of lipoxygenase, which is a biological reaction (food chemistry). It is the free radical mechanism that is best explained and studied. The initial phase of this mechanism is not fully explained, but it is believed that it is the cause of photo-oxidation. The first product in autoxidation reactions is hydroperoxide, which is more stable than other products. This is why it is used in the evaluation of lipid peroxidation in foods. Secondary products formed as a result of oxidation are variable. These include: aldehydes, ketones, epoxides, hydroxy compounds, even oligomers and polymers [1] (Figure 1).

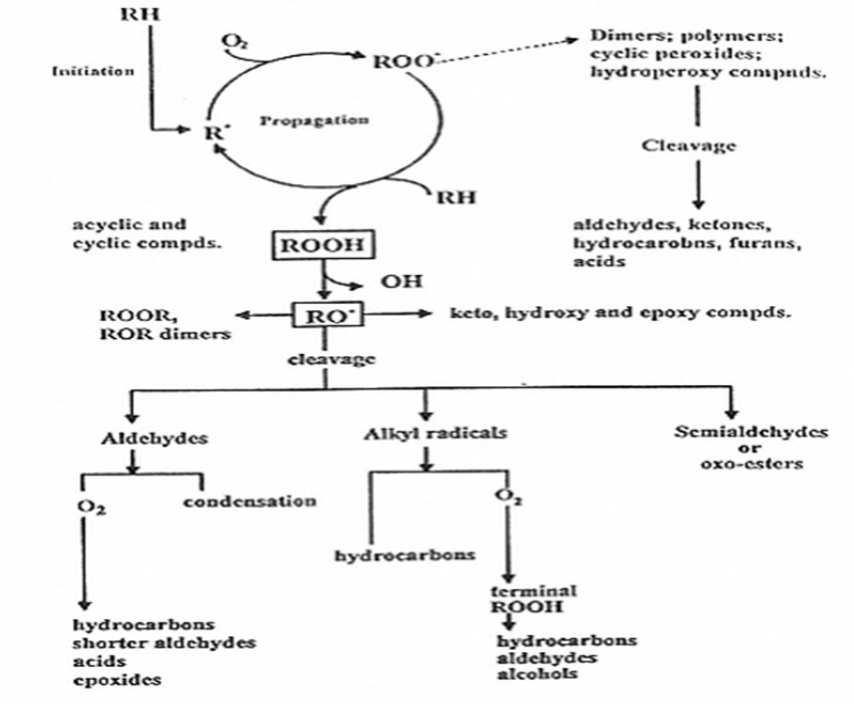


Fig. 1 Generalized scheme for autoxidation of lipids [2].

The methods used to measure lipid oxidation in foods are aimed at either primary products or secondary products. Therefore, the most commonly used methods are peroxide value, Thiobarbituric acid test, Kreis test and Iodine value, although there are many tests related to lipid oxidation measurement in the literature [3].

Among these, the costs of the highest specifity (NMR and EPR) are very high. On the other hand, there are methods (IR and Raman scattering) that require precision at the point of application and labor, even though the cost is low (Figure 2) [3]. Among all methods, TBA method is the most practical and widely used method at both cost and application point of view.

TBA assay first used for detection of oxidation products in animal tissue [4]. After that, its first usage in food was to detect oxidation of milk fat [5]. Patton and Kurtz [5] at this research found that malonaldehyde (MDA) was the product of rancidity process and can be used for detection and measuring of lipid peroxidation by TBA assay.

It is well known that MDA is produced in the last step of the autoxidation of unsaturated oils and fats. Besides, they indicated that TBA assay is more sensitive than the Kreis test and peroxide value. Dunkley and Jennings [6] were also tested the sensitivity of TBA assay. With respect to their research, TBA assay is reproducible and more sensitive than the Kreis test.

Traditional foods are special products having cultural aspects of the local regions of the countries. Manti (stuffed pasta) is one of the famous and nutritious traditional foods in Turkey and Middle Asia. Manti is sensitive to oxidation and microbial deterioration because it contains minced meat and dough [7]. Therefore, the aim of the study was to find correlation between lipid peroxidation and its antimicrobial effect. For this purpose *Salmonella typhimerium* and *E.coli* 0157: H7 were used as sample microorganisms.

METHOD	ANALYTE	SAMPLE PREPARATION	AMOUNT OF SAMPLE	SENSITIVITY	SPECIFICITY	COST	LIMITATIONS
Titration	Peroxides	Medium-Short	1 g	Medium-low	Medium-low	Low	Reagents susceptible to oxidation Absorption by UFA Dryness required
Uv-Vis[a] spectroscopy	Peroxides, *Conjugated dienes/trienes *MDA, aldehydes	Medium	500 mg	Medium	Medium	Low	High amount of solvents Low concentration range Variability depending on the dye *Insensitive to oleic acid
Chromatography	Peroxides, MDA, SOPs, volatiles, oligomers	Long	1-100 mg	High-very high (depending on the detector)	High-very high (depending on the detector)	High	Laborious experimental procedure and data processing
Chemiluminiscence	Peroxides	Short	1-200 mg	High	Medium	Low	Unknown mechanisms Light amplifiers required
Fluorescence	Aldehydes and volatiles	Very short	10-50 mm^2	Very high	High	Medium	Variability in wavelenghts
IR[b] spectroscopy	Peroxides, unsaturations, MDA	Very short-none	2-40 mg	Medium-high	High	Medium	Non-aqueous solutions required
Raman scattering	Peroxides, unsaturations, MDA	Very short-none	10-50 mm^2	Medium-high	High	Low	Some molecules are inactive
Nuclear magnetic resonance	Peroxides, aldehydes, dienes	Very short-none	10-200 mg	High	Very high	Very high	Complex data interpretation
Electron paramagnetic resonance	Radicals	Very short-none	100-900 mg	High	High	Very high	Complex data interpretation

[a] Ultraviolet-visible
[b] Infrared

Fig. 2 Characteristics of the different methods for analysis of lipid oxidation in foods reviewed in this article.

2. Material and Method

2.1 Thiobarbituric acid (TBA) analysis

Manti sample (10 g) was homogenized with water. The mixture was transferred to Kjeldahl flask and distilled by adding 2.5 mL 4 N HCl (Merck, Germany) and 1 mL antifoam chemical. After that, 5 mL of this distillate was mixed with equal volume of TBA (Merck, Germany) and incubated in water bath at 80-90 ^{o}C for 30 minutes. The measurement was made on a spectrophotometer at 538 nm and lipid peroxidation level was calculated according to below equation as equivalent of mg malondialdehit (MDA) per kg sample [8].

2.2 Antimicrobial activity

The manti sample distillates were used for their antimicrobial activity and evaluated by the help of disc diffusion method. The antimicrobial activity was tested against *E. coli O157:H7* ATCC 33150 and *S.typhimerium* as food-borne pathogens (kindly provided by Kayseri Agriculture Control Protection Management Center, Turkey). Strains first incubated overnight in Mueller-Hinton broth at 35 °C. After that, culture turbidity was adjusted to 0.5 McFarland standard and inoculated on to Mueller-Hinton agar by spread plate technique. Paper discs saturated with 20 µL of distillates were used for antimicrobial activity. The zones of inhibition on MH Agar were measured after 24 hour incubation at 35^{o}C [9].

3. Results and Discussion

Antimicrobial activity of lipid peroxidation was tested through this study. MDA determination as an indication of lipid peroxidation by the TBA assay can offer, at best, a narrow and somewhat empiric products of lipid peroxidation and the TBA assay is perhaps the most widely used assay for oxidative damage. Therefore, TBA assay was used for lipid autoxidation determination. The results showed that increase in lipid peroxidation level concentration resulted in increase in antibacterial activity (Figure 3). If the lipid peroxidation value in distillates was 1.5 mg/kg and above, it showed antimicrobial activity. Thus, it can be said that through storage, presence of TBA may inhibit bacterial growth and contamination.

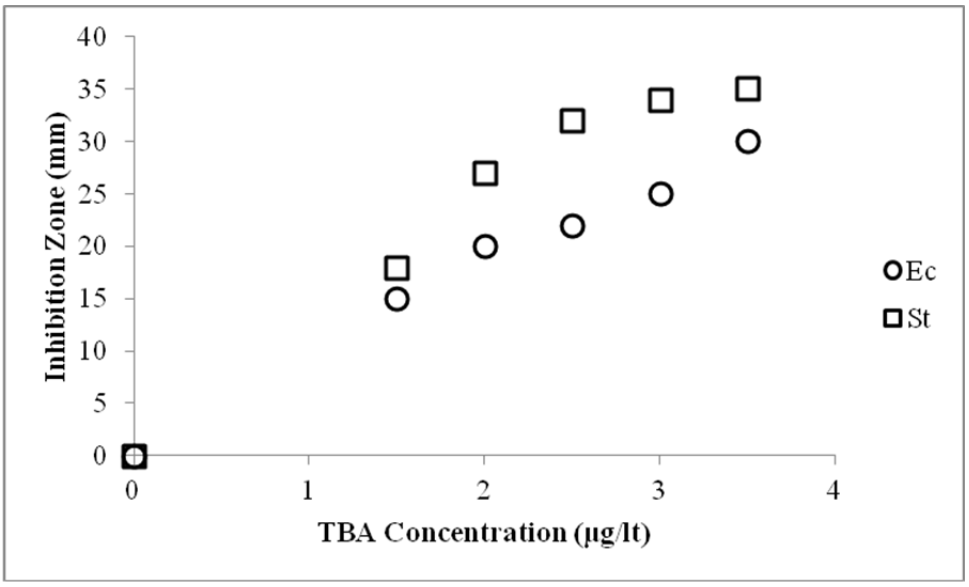
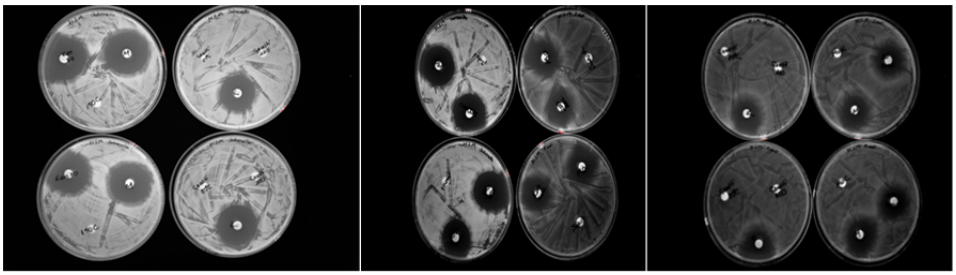


Fig. 3 Diameter zones for some pathogens related to TBA concentration [Ec: *E.coli* zone diameter(mm) St: *S.typhimerium* zone diameter(mm)]. *Control group was used in the study. **The zones formed by *S. typhimurium* and *E. coli* showed in Annex-1.

Although the TBA value of food products greater than 3 mg / kg is correlated with malodor and taste in foods [10,11], deterioration resulted from lipid peroxidation in Manti is not directly correlated with lipid peroxidation level. Some studies indicated that there was no reduction in food quality with respect to increasing lipid autoxidation. Besides, Yücetepe [12] found that sensory quality of manti increased with increasing lipid peroxidation level.

As a conclusion, lipid peroxidation level in Manti cannot express applicable value in the deterioration of manti. Therefore, there is a need for further research on this subject.



Annex 1 The zones formed by *S. typhimurium* and *E. coli*.

References

[1] Guillen-Sans R, Guzman-Chozas M. The thiobarbituric acid (TBA) reaction in foods: a review. Critical reviews in food science and nutrition. 1998 ; 38 (4) : 315-350.

[2] Fennema Owen R, editor. Food Chemistry 3rd. New York: Marcel Decker; 1996. Available from: https://www.abebooks.com/Food-Chemistry-3rd-Edition-Owen-Fennema/9499373701/bd

[3] Barriuso B, Astiasarán I, Ansorena D. A review of analytical methods measuring lipid oxidation status in foods: a challenging task. European Food Research and Technology. 2013 ; 236(1) : 1-15.

[4] Kohn H I, Liversedge M. On a new aerobic metabolite whose production by brain is inhibited by apomorphine, ergotamine, epinephrine, and menadione. J. Pharmacol. 1944; 82-292.

[5] Patton S, Kurtz GW. 2-Thiobarbituric acid as a reagent for detecting milk fat oxidation. J. Dairy Sci. 1951; 34: 669.

[6] Dunkley W L, Jennings WG. A procedure for application of the thiobarbituric acid test to milk. J. Dairy Sci. 1951; 34: 1064.

[7] Gökmen S, Aydın MF, Kocabaş A, Sayaslan A, Yetim H. Çağlar A. (. A study on quality criteria of commercial stuffed pasta (mantı), as traditional food. International Food Research Journal. 2015; 22 (5):2018-2024.

[8] Uzunlu S. Modified atmosphere packaging of manti with different gas compositions and its microbial and chemical composition variances during cold storage. Çukurova University Institute of Natural and Applied Sciences Department of Food Engineering. PhD Thesis. 2011; 140 p.

[9] Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiological reviews. 2007; 87(1) : 315-424.

[10] Soldatou N, Nerantzaki A, Kontominas MG, Savvaidis IN. Physicochemical and Microbiological Changes of "Souvlaki"– A Greek Delicacy Lamb Meat Product: Evaluation of Shelf-Life Using Microbial, Colour and Lipid Oxidation Parameters. Food Chemistry. 2009 ; 113 (1) : 36-42.

[11] Yılmaz I, Demirci M, Effect of Different Packaging Methods and Storage Temperature on Microbiological and Physicochemical Quality Characteristics of Meatball. Food Science and Technology International. 2010 ; 16 (3) : 259-265.

[12] Yücetepe A. 2011. The effects of modified atmosphere packaging on the quality of manti (a meat-filled pasta product) Istanbul Technical University Institute of Natural And Applied Sciences Department of Food Engineering. Master Thesis. 2011; 87 p.

Moroccan sesame: an overview of seed and oil quality

Abdelghani Nabloussi[1], **Hafida Hanine**[2], **Meriem El Harfi**[1,2] and **Hajar Rizki**[1,2]

[1] Research Unit of Plant Breeding and Plant Genetic Resources Conservation, National Institute for Agricultural Research, Regional Center of Meknes, Meknes, Morocco
[2] Laboratory of Bioprocess and Bio-Interfaces, Faculty of Science and Technics, University of Sultan Moulay Slimane, Beni Mellal, Morocco

Sesame (S*esamum indicum*.L) is one of the most valuable oilseed crops whose seeds are used as traditional health food, with high quality oil, protein and natural antioxidants. Many researches were carried out to characterize, for the first time, the Moroccan sesame seeds (raw and roasted) for major and minor compounds that play a crucial role in oil quality and stability. They contained high amount of protein (26-28%), considerable amount of oil (45-55%), characterized by an elevated level of unsaturated fatty acids (79.50-83.40% of UFA) and high content of stearic acid (7.3-8.6%), and remarkable content of dietary fibers. Seed oil was also found to be rich in total phenolic content, chlorophyll content and carotenoid content, with average values of 52.37, 3.51 and 1.71 mg/kg oil, respectively. The specific extinctions (K232) and (K270) ranged from 2.86 to 6.49 and from 0.62 to 2.13 respectively. It was showed that sesame seeds can be considered as a good source of natural antioxidant, particularly after roasting. Due to their distinguishable and favorable properties, Moroccan sesame seeds could be useful for food, industrial and pharmaceutical purposes.

Keywords: Sesame; Morocco; biochemical characterization; antioxidant; food

1. Introduction

Being one of the earliest domesticated plants in the world, sesame is a very ancient oilseed crop, well adapted to the tropics and subtropics [1]. It has been cultivated for centuries, particularly in Asia and Africa, for its high content of edible oil and protein. India and China are, actually, the largest producers of sesame in the world, followed by Myanmar. Sesame plant has received considerable attention around the world due to its seeds used for oil extraction and food preparations [2]. It ranks second, after olive oil, with regard to nutritional value [3]. Sesame seeds have the highest oil content compared to rapeseed, peanut, soybean, and other oil crops [4]. It has an important place in human nutrition, medicinal, pharmaceutical, industrial and agricultural uses. Its oil has a high stability and resistance to oxidative deterioration due to different bioactive molecules like phenolic compounds, lignans such as sesamin, sesamol, sesaminol and α-tocopherol [5], improving food quality and antioxidative stability.

Africa grows more than 50% of the world's sesame production [6]. In Morocco, the average production is about 1800 t/year. Tadla area ensures 90% of national production, whilst Meknes and Safi areas provide the rest [7]. This sector remains weak because of several constraints, such as lack of improved varieties, use of traditional production techniques and low valorization of production. One key to valorize the production is to know the agronomic performance and the quality indexes of the vegetal material cultivated. In this context, many researches were carried out to characterize, for the first time, the Moroccan sesame seeds (raw and roasted), belonging to 13 local cultivars, for major and minor compounds that play a crucial role in oil quality and stability. In this chapter, findings of these researches will be presented and discussed in light of the results previously reported in other regions of the world.

2. Morphological characterization of Moroccan sesame seeds

Moroccan sesame seeds were characterized by spherical, flat, large oval, oval and narrow oval form, while the coat color, which is a relevant parameter determining seed quality, varied from yellow to brown. However, a large variability in the color of seed coat, ranging from black to white and intermediate colors (e.g. brown or yellow), was reported [8]. Seed size was very variable, and one could observe that seed length, width and thickness ranged from 2.3, 1.3 and 0.71 mm, respectively, to 3.2, 2 and 0.87 mm, respectively. These results are comparable with those found by Seemaparoha et al. [9] for Indian sesame (2.95 mm, 1.85 mm and 0.84 mm, respectively for seed length, width and thickness).

The value of thousand seeds weight (TSW) of Moroccan sesame ranged from 2.72 to 3.27 g, with an average value of 3.05 g. This finding is in accordance with that of El Khier et al. [10] in Sudanese sesame (2.33-3.70 g) and that of Abdou et al. [11] in Nigerian sesame (2.49-5.5 g, with an average TSW of 3.2 g). On the other hand, the Moroccan sesame TSW was found to be higher than that of Vietnamese and Cambodian sesame (2.84) [12] and Pakistani sesame (2.6 g) [13], whilst it was lower than that of Turkish sesame, having an average TSW of 3.7 g [14].

3. Biochemical characterization of Moroccan sesame seeds

Sesame seeds protein contains high amounts of aspartic acid, glutamic acid and arginine [15], presenting a great reservoir of amino acids. Also, sesame seeds are rated among the highest organic sources containing cysteine [16]. The protein content depends on the climatic conditions as well as the stage of development of the plant. The protein content of the 13 Moroccan cultivars grown in different areas varied from 26.77 to 27.93%, with an average of 27.4% (Table 1). These values are higher than those found by Elleuch et al. [2] (25.77%), Moazzami et al. [17] (24.55%) and Borchani et al. [18] (24.63%). These differences could be attributed to either sesame varieties or environments where they grow. Also, sesame seeds are an important source of dietary fiber. The Moroccan cultivars contain high amounts of dietary fiber, ranging from 17.55 to 20.84% of dry matter, with an average of 19.2%. The amount of insoluble dietary fibers varied from 12.46 to 15.78%, while that of soluble dietary fibers varied from 4.50 to 5.87% (Table 1). Average of the latter was 5.21%, which is higher than that of cereals and its derivatives, such as corn, wheat bran, oat bran and rice bran, having a fiber content ranging from 0.4 to 4.1% [19]. Thus, sesame seeds can be considered as a good source of fiber that could be used in food formulations.

Table 1 Nutrients variation in Moroccan sesame seeds.

	Minimum	Maximum	Average
Protein (%)	26.770±0.670	27.930±0.060	27.400±0.540
Insoluble fiber (%)	12.460±0.500	15.780±0.200	14.190±0.400
Soluble fiber (%)	4.500±0.090	5.870±0.300	5.210±0.200
Total fiber (%)	17.550±0.100	20.840±0.150	19.200±0.210
Phenolic content(mg/g)	3.750±0.050	3.920±0.030	3.860±0.090
Flavonoïdes content (mg/g)	0.130±0.003	0.140±0.006	0.135±0.009

Polyphenols, which are secondary metabolites distributed in the plant, are considered to be very important antioxidants, due to their ability to give a hydrogen atom or an electron to form an intermediate stable radical, and consequently prevent the oxidation of different biological molecules. The polyphenol content of Moroccan sesame seeds ranged from 3.75 to 3.92 mg/g (Table 1). The average of this content was 3.86 mg/g, higher than that of Vishwanath et al. [20] which reported a value of 2.88 mg/g. The seeds variety and origin as well as the cultivation zone greatly influence the polyphenol content. The polyphenol content of sesame seeds is higher than those of banana (2.32 mg/g) and carrot (1.52 mg/g) [21]. The content of flavonoids, which are also secondary metabolites, with an important role in the plant defense system, and which function as hydroxyl radical sensors and peroxides, ranged from 0.13 to 0.14 mg/g (Table 1), and no significant differences were observed between the cultivars. Actually, the content of primary and secondary metabolites is strongly influenced by plant stress when exposed to severe drought conditions.

Sesame is also considered as a rich source of minerals such as calcium, magnesium (which plays an important role for the support of the respiratory system), iron, potassium and selenium which is detected in sesame at doses beneficial to health [22]. Table 2 combines the results of the determination of macro and microelements in Moroccan sesame seeds: calcium, phosphorus, potassium, magnesium, selenium, iron and zinc. Contents variation in the studied cultivars was 928-997 mg/100 g for calcium, 404-598 mg/100 g for phosphorus, 467-532 mg/100 g for potassium, 317-389 mg/100 g for magnesium, 14.90-15.90 mg/100 g for iron, 5.64-5.97 mg/100 g for zinc and 51.08-61.19 µg/100 g for selenium. These results are very similar to those reported for sesame from other countries, such as Tunisia [23] and Turkey [24].

Table 2 Minerals content variation in Moroccan sesame seeds.

	Maximum	Minimum	Average
Calcium (mg/100 g)	997	928	971.14
Phosphorus (mg/100 g)	598	404	545.83
Potassium (mg/100 g)	532	467	493.57
Magnesium (mg/100 g)	389	324	347.40
Iron (mg/100 g)	15.90	14.90	15.33
Zinc (mg/100 g)	5.97	5.64	5.79
Selenium (µg/100 g)	61.19	51.08	55.99

Calcium, phosphorus and potassium are the most abundant minerals in seeds of the different cultivars. Magnesium, which is an essential mineral for enzyme activity, is also found in considerable amount in sesame seeds. Like calcium, magnesium plays a role in regulating acid-alkaline balance in the body, and a significant role in photosynthesis. Also, the potassium is an essential nutrient and has an important role in the synthesis of amino acids and proteins [25]. Our

results have confirmed that sesame is a very good source of minerals, especially calcium, phosphorus and selenium, which have a healthy nutritional and pharmaceutical role.

4. Physicochemical and biochemical characterization of Moroccan sesame oil

Oil content is the most important characteristic of oilseed crops. It varied for Moroccan sesame, from 45% to 55%, with an average value of 48.24% (Table 3). These findings are in agreement with those reported in other studies, such as that of Nzikou et al. [26]. However, Abdullahi et al. [27] had reported that oil content of different sesame cultivars ranged from 50% to 69.03%, with an average of 59.5%, whilst Baydar et al. [28] had found higher average oil content of 63.25% in Turkish cultivars. Variation in oil content can be attributed either to varietal factor, environmental factor, or interaction of both factors. High oil content recorded in Moroccan cultivars (over 50%) is a desirable trait for breeding programs to improve sesame cultivars.

Quality parameters used regularly to measure the physical and chemical properties of edible oils are content of free fatty acid (FFA), peroxide value, iodine value and saponification value. The FFA of Moroccan sesame oils varied from 0.12 to 0.60% of oleic acid (Table 3). The recorded values were lower than those found by Ogbonna et al. [29] (0.25-1.41% of oleic acid), Borchani et al. [18] (1.64% of oleic acid), Dim [30] (5.54% of oleic acid) and Weiss [31] (1.90 to 2.00% of oleic acid). The maximum acceptable value for sesame oil recommended by the Codex Alimentarius Commission for oils seeds is 4% of oleic acid [32] and the maximum value as proposed by FAO is 6 mg KOH/g oil. The high acid value is frequently an indication of a strong enzymatic hydrolysis of seed oils during harvesting, handling or oil processing [33]. The saponification index of sesame oil from different locations in Morocco varied significantly among the studied cultivars, from 82.52 to 179.52 mg KOH/g of oil (Table 3). These values are slightly lower than those reported in other previous studies, (192 mg KOH/g of oil) [26] and (190.74 mg KOH/g of oil) [30]. Peroxide value is an indication of rancidity and it is the most important indicator of the stability of edible oils [34]. Therefore, a high peroxide value indicates a poor resistance of oil to peroxidation during storage. The peroxide values of Moroccan sesame oils ranged from 1.7 to 4.17 meq O_2/kg oil (Table 3), which are below the maximum acceptable value of 10 meq O_2/kg set by the Codex Alimentarius Commission [32]. These values are consistent with those reported by Ogbonna et al. [29] and Dim [30], and higher than those found by Borchani et al. [18]. These results suggested that sesame oil stability to oxidation is relatively good, which is due to the presence of antioxidants. The iodine value is a measure of the total number of double bonds present in fats and oils [33]. Iodine values of Moroccan sesame oils ranged from 82.9 to 156.85 g of I_2/100 g oil (Table 3). These results are in agreement with those found in previous studies [29; 30; 18]. The iodine value recorded was higher in all cultivars, indicating a higher concentration of unsaturated fatty acid in the Moroccan sesame cultivated in various locations.

Table 3 Variation in physicochemical and biochemical parameters of Moroccan sesame oil.

Parameters	Minimum	Maximum	Average
Oil content (%)	45±2.76	55±2.87	48.24
Acidity (% of oleic acid)	0.12±0.01	0.6±0.13	0.26
Peroxide index (meq O_2/kg)	1.7±0.29	4.17±0.29	2.09
Saponification Index (mg KOH/g)	82.52±1.40	179.52±2.81	141.74
Iodine Index (g of I_2/100 g)	82.9±4.88	151.18±4.76	113.75
Unsaturated fatty acid (UFA) (%)	79.50±1.78	82.34±1.85	81.11
Ratio C18:1/C18:2	0.79±0.049	0.97±0.051	0.91
Phenolic content (mg GAE/kg)	46.30±0.05	60.12±0.03	52.37
Chlorophyll (mg of pheophytin /kg)	0.53±0.05	7.57±0.20	3.51
Carotenoid (mg of lutein /kg)	0.59±0.01	3.24±0.11	1.71

Hydroperoxide and the conjugated diene reflect the degree of formation of primary products of lipid oxidation [35]. The higher concentration of conjugated dienes and trienes induce greater amounts of coefficient of extinction K232 and K270. The K232, which measures the amount of conjugates dienes, varied between 2.03 and 3.54. The secondary oxidation compounds of oils evaluated by measuring the extinction coefficient at 270 nm (K270) recorded values ranging from 0.89 to 2.13. These reported values are consistent with those reported by Elleuch et al. [2], for sesame, close to those found by Abdalla et al. [36], for olive oil (K232: 2.86-3.45 and K270: 0.32-0.62) and higher than those reported by Gharby et al. [37] for argan oil (K232: 1.02-1.49 and K270: 0.18-0.25). At the same peroxide value, the K232 and K270 for sunflower, olive, and the pumpkin seed oils were reported to be 4.93 and 0.51, 3.32 and 0.65, and 8.88 and 1.99, respectively [38].

Moroccan sesame oils have been also characterized by high unsaturated fatty acid (UFA) witch varied from 79.50 to 82.34% (Table 3), with close contents of oleic and linoleic acids. High level of UFA increases the oil quality, allowing this oil to be suitable for human consumption. The ratio C18:1/C18:2 ranged from 0.79 to 0.97, with an average value

of 0.91 (Table 3). The mean value of this ratio was similar to those reported by Codex Alimentaruis and for the sesame from Egypt, Turkey, Congo and Sudan [10; 26; 39; 40]. The variation observed among the cultivars grown in different locations might be due to both genotypic effect and the difference in environmental conditions, especially the temperature [41; 42]. The ratio oleic/linoleic for sesame seeds oil was higher than those of olive (0.03), sunflower (0.26), soybean (0.43) and corn (0.5), but lower than those of rapeseed (2.89), peanut (1.68), flax (1.21), coprah (4) and plam oil (3.8) [43]. Besides, the Moroccan sesame was characterized by a quite high content of stearic acid, varying from 7.30 to 8.60%. These are the highest values ever reported, in comparison to contents found in other parts of the world. In fact, analyzing fatty acids composition of a world sesame collection, a variation of 3.40-6.00% in stearic acid content was found [44]. More recently, a range of 2.10-4.80% was reported in Turkish sesame [45], whilst an average of 5.80% was reported in Sudan [18]. Therefore, high stearic acid content could be taken as indicator or marker of Moroccan sesame authenticity. Contrarily to other predominant saturated fatty acids (SFA), like as palmitic acid, mystiric acid and lauric acid, which increase total cholesterol in human blood, the stearic acid is known to have a neutral effect on total and LDL cholesterol [46; 47]. Furthermore, oils with elevated stearic acid enable the production of solid fat without need of hydrogenation [48]. Therefore, such oils could be very interesting for food industry.

The total phenolic content of sesame oil extracts ranged from 47 to 60 mg GAE/Kg of oil (Table 3). This difference may be due to extraction techniques of oil, environmental and ecological characteristics of the particular growing area [2]. These values are much higher than those formerly found in other regions of the world, 23 mg GAE/Kg [2] and 14.21 mg GAE/Kg [18]. Overall, sesame oil extracts contained higher total phenolic content compared to other commonly available vegetable oils [49].

Chlorophyll and carotenoids are important quality parameters because they have a correlation with color, which is a basic attribute for evaluating oil quality. Their magnitude depends on different factors, such as fruit ripeness, cultivar, climate conditions, type of soil, and extraction procedures. Moroccan sesame oil exhibited a notable amount of carotenoids ranging from 0.59 to 3.34 mg/kg of oil and chlorophylls ranging from 0.53 to 7.57 mg/kg of oil (Table 3), which are responsible for the yellow color of the seed oil.

The obtained values are higher than those reported by Borchani et al. [18] for raw sesame oil (0.04 mg/Kg of oil, for chlorophyll, and 2.62 mg/kg oil, for carotenoids). The average chlorophyll content recorded in Moroccan sesame cultivars was found to be higher, compared to other vegetable oils. The content of chlorophyll of sunflower, date palm and Moroccan olive is 0.99, 2.18 and 1.69 mg/kg of oil, respectively [50; 51; 52].

5. Effect of roasting on sesame antioxidant compounds and index parameters

Sesame oil prepared from roasted sesame seeds has a characteristic odor and taste and longer shelf life, compared to unroasted seeds, which give it higher oxidative stability than other vegetable oils. Its remarkable stability is due to the presence of a large quantity of endogenous antioxidants. Roasting conditions of temperature and time have a great effect on color and quality of sesame seeds and oils, causing some desirable or undesirable changes in physical, chemical and nutritional properties of the seeds. One of the desired outcomes of roasting process is the increase in antioxidant activity that occurs as a result of the formation of Maillard reaction products [53]. However, establishing the optimum level of roasting conditions, leading to good quality of sesame oil, is one of the main problems for oil sesame producers.

We evaluated phenolic, lignans, antioxidant activity and index of quality of sesame oil from roasted and unroasted seeds, because those compounds are believed to be bioavailable and bioactive.

Table 4 Variation in some antioxidant compounds of oil from different times roasted sesame seeds.

Samples	Sesamol (mg/kg)	Sesamin (mg/kg)	Antioxidant activity (%)	Phenolic compound (mg/kg)	Flavonoids (mg/kg)
Control	57.52±0.50	57.27±0.90	60.98±0.90	86.7±0.85	0.092±0.002
30 min	66.69±0.80	61.41±0.70	61.2±0.70	87.4±0.65	0.096±0.003
60 min	68.73±0.40	61.43±0.80	62.5±0.80	87.45±0.55	0.096±0.003
90 min	69.99±10	61.69±0.97	63.5±0.97	87.55±0.57	0.096±0.003
120 min	71.6±0.95	62.18±1.00	60.69±1.00	86.6±0.50	0.095±0.003
150 min	71.61±0.75	53.14±2.50	60.59±2.50	86.57±0.50	0.092±0.002
180 min	69.46±1.50	51.17±3.00	59.57±3.00	84.47±0.56	0.082±0.003
210 min	66.07±0.75	46.43±1.80	59.5±1.80	82.33±0.50	0.072±0.002
240 min	49.15±0.85	46.77±1.00	53.17±1.00	81.17±0.45	0.067±0.002
270 min	47.08±0.96	46.87±2.50	48.72±2.50	80.72±0.65	0.063±0.001
300 min	46.39±1.02	46.19±3.00	47.09±3.00	78.09±0.45	0.058±0.003
330 min	39.91±0.39	39.73±1.00	46.11±1.00	75.11±0.43	0.053±0.002
360 min	35.49±0.87	35.33±2.00	45.98±2.00	73.98±0.46	0.05±0.001

The results showed that the antioxidant activity increased significantly as the roasting temperature was fixed at 150°C during the first 90 min, with a mean value of 63.50%, compared to 60.98% observed for unroasted sesame (Table 4). The increase in antioxidant activity could be related to the change occurred in natural antioxidants phenolic compounds in roasted seeds. It was reported that antioxidant activity and amount of total phenolic compounds increased significantly as the roasting temperature and time rose up until 200°C during 20 min, and then decreased by roasting at 220°C. The highest antioxidant activity and phenolic compounds content were obtained by roasting at 200°C for 20 min [54].

The oxidative stability of sesame oil is higher than that of other vegetable oils, which is due to the presence of a large quantity of endogenous antioxidants and phenolic compounds, comprising sesamin, sesamolin, sesamol, and γ-tocopherol [55]. Also, the relatively greater oxidative stability of oils from roasted sesame may result from the formation of some new antioxidants. There was also an increase in phenolic content and flavonoids when seeds were roasted at 150°C during the first 120 min. The highest increase was observed for 90 min, with average values of 87.55 mg/kg and 0.095 mg/kg, respectively for phenolic and flavonoids content, compared to 86.70 mg/kg and 0,092 mg/kg, observed for unroasted seeds (Table 4). Our results are in agreement with those obtained by Jeong et al. [56] who showed that antioxidant activity of defatted sesame meal extracts was affected by roasting conditions. The roasting treatment for 2 hours at 150°C was also very promoting for the lignans contents, with more pronounced oil color and flavor. The average sesamin content increased up to 62.69 mg/kg, compared to 57.27 mg/kg, for unroasted seeds sample (Table 4). Sesamol, which is a potent phenolic antioxidant, was detected in low amount in raw sesame oil, 57.52 mg/kg. However, this content was much higher than that reported in other studies [57; 58]. By roasting, the sesamol content increased remarkably, due to sesamoline hydrolysis during the thermal oxidation. After roasting at 150°C, during 150 min, sesamol content rose up to 71.61 mg/kg. Similar results had been found in other research [59]. It was demonstrated that this component has an important preventive effect against the thermal decomposition of tocopherol [60].

Regarding the quality index, we also observed differences between roasted and unroasted sesame oils, for all the index parameters studied (Table 5). During the first 120 min of roasting, these parameters remained unchanged, indicating the high quality of oils and their stability. The iodine index showed a high stability during 150 min of roasting (Table 5), which confirmed that these oils are highly unsaturated, suggesting high levels of oleic and linoleic acids [2] that decrease with the increase of time of roasting. The saponification index of raw sesame was lower than that of roasted samples which increased with the time of roasting.

Table 5 Index parameters of oil from different times roasted sesame seeds.

Samples	Iodine index (I_2/100 g)	Saponification index (mg KOH/g)	Peroxide index (meq O_2/kg)	Extinction index	Acidity index (mg KOH/g)
Control	110.90±0.85	189±1.91	10±0.14	1.46±0.04	0.6±0.09
30 min	110.82±1.92	189±2.05	10±0.22	1.46±0.02	0.6±0.07
60 min	110.74±1.81	189±1.47	10±0.21	1.46±0.01	0.6±0.08
90 min	110.66±1.11	189±2.01	10.20±0.35	1.46±0.01	0.6±0.10
120 min	110.56±2.01	190±0.98	10.30±0.33	1.46±0.03	0.6±0.21
150 min	109.96±2.50	193±0.75	11.40±0.23	1.47±0.03	0.6±0.10
180 min	103.87±1.75	193.75±1.03	11.78±0.54	1.65±0.04	0.8±0.07
210 min	100.38±2.09	193.99±0.89	11.98±0.43	1.70±0.03	1.3±0.10
240 min	96.26±1.22	197±1.50	12.03±0.33	1.79±0.03	1.27±0.10
270 min	94.46±1.01	197.65±1.67	12.56±0.52	1.82±0.02	1.25±0.10
300 min	87.18±1.50	197.88±1.47	12.87±0.29	1.93±0.04	1.3±0.00
330 min	86.82±1.22	199.50±1.44	13.20±0.53	1.99±0.03	1.32±0.15
360 min	85.70±1.90	200.40±1.97	13.50±0.34	2.10±0.04	1.90±0.20

From 150 min of roasting, one could observe an increase in the acidity, saponification, peroxide and specific extinction. This might be explained by the effect of roasting process leading to higher content in primary oxidation products. On the contrary, the iodine index showed a decrease after 2 hours of roasting, which might be explained by the effect of roasting process on the formation of unsaturated fatty acids in sesame oil.

The roasting processing changed remarkably polyphenol, lignans, antioxidant activity and quality index of sesame oil. To obtain the highest antioxidant activity and high amounts of bioactive compounds from sesame oil, seeds should be roasted for 2 hours at 150°C. The sesame seed and oil showed a high stability facing high temperature roasting, suggesting that sesame oil could be utilized as a potential source of edible oils for human consumption and could be incorporated into a normal diet at a level that might benefit health as a natural antioxidant.

6. Conclusion

The present review summarized the results of some studies on Moroccan sesame characterization, which were carried out for the first time in Morocco. Major and minor compounds of oil as well as raw and roasted seeds were analyzed for various local cultivars from the Tadla area that ensures around 90% of the national sesame production. Large variability was observed for most of the compounds analyzed, indicating a high genetic diversity among the cultivars. Overall, these cultivars were interestingly characterized by a high amount of protein, an elevated content of stearic acid and a high yield of dietary fibers. Therefore, high stearic acid content could be taken as marker of Moroccan sesame authenticity. Also, the Moroccan sesame seed oil was found to be rich in total phenolic content, chlorophyll content and carotenoid content. Furthermore, there was a decrease in the iodine value and an increase in others parameters, such as acid value, saponification value, peroxide value and specific extinction coefficient, by increasing roasting time. Thus, sesame seeds can be considered as a good source of natural antioxidant, particularly after roasting. The obtained results suggested phenolic and flavonoid contents and lignans were the main responsible for the antioxidant potential of sesame. Due to their distinguishable and favorable properties, Moroccan sesame seeds could be useful for food, industrial and pharmaceutical purposes. Besides, the observed genetic diversity for most of the parameters studied open up the possibility of breeding Moroccan sesame for combining high seed yield and high seed and oil quality.

References

[1] Ashri A. Sesame (*Sesamum indicum* L.). In: Singh RJ, (Ed.). Genetic Resources, chromosome engineering, and cropimprovement. New York: CRC Press, Taylor & Francis Group; 2007. P. 232-289.

[2] Elleuch M, Besbes S, Roiseux O, Blecker C, Attia H. Quality characteristics of sesame seeds and by products. Food Chemistry. 2007; 103:641-650.

[3] Alpaslan M, Boydak E, Demircim M. Protein and oil composition of soybean and sesame seed grown in the Harran (GAP) area of Turkey. Session 88B, Food Chemistry: Food Composition and Analysis. IFT Annual Meeting - New Orleans; 2001.

[4] Anilakumar KR, Pal A, Khanum F, Bawa AS. Nutritional, medicinal and industrial uses of sesame (*Sesamum indicum* L.) seeds-an overview. Agriculturae Conspectus Scientificus. 2010; 75 (4):159-168.

[5] Lee J, Lee Y, Choe E. Effects of sesamol, sesamin and sesamolin extracted from roasted sesame oil on the thermal oxidation of methyl linoleate. LWT FoodSci. Tech. 2008; 41:1871 -1875.

[6] FAOSTAT, 2014. World statistics on sesame area, yield and production. Available from: (www.fao.org/faostat/en/#data/QC).

[7] PMV, 2008. Green Morocco Plan Agricultural Staretgy (Plan Maroc Vert). Agricultural Regional Plan of Tadla-Azilal Region.

[8] Zhang H, Miao H, Wei L, Li C, Zhao R, Wang C. Genetic analysis and QTL mapping of seed coat color in sesame (*Sesamum indicum* L.). PLOS ONE. 2013; 8(5): 1-10.

[9] Seemaparoha R, Prakash M , Varsha T. Impact of Present Climate on Physical and Biochemical Properties of Two Sesame (*Sesamum indicum* L) Cultivars. International Journal of Engineering Science and Computing. 2016; 6: 7726- 7732.

[10] El Khier MKS, Ishag KEA, Yagoub AEA. Chemical composition and oil characteristics of sesame seed cultivars grown in Sudan. Research Journal of Agricultural Biological Science. 2008; 4(6):761 -766.

[11] Abdou RIY, Moutari A, Ali B, Basso Y, Djibo M. Variability Study in Sesame (*Sesamum indicum* (L)) Cultivars based on AgroMorphological Characters. International Journal of Agriculture, Forestry and Fisheries. 2015; 3(6): 237-242.

[12] Pham TD, Geleta M, Bui TM, Bui TC, Merker A, Carlsson AS. Comparative analysis of genetic diversity of sesame (*Sesamum indicum* L.) from Vietnam and Cambodia using agro-morphological and molecular markers. Hereditas, 2011; 148: 28-35.

[13] Akbar F, Rabbani MA, Shinwari ZK, Khan SJ. Genetic divergence in sesame (*Sesamum indicum* L.) landraces based on qualitative and quantitative traits. Pakistan Journal of Botany. 2011; 43: 2737-2744.

[14] Furat S, Uzun B. The use of agro-morphological characters for the assessment of genetic diversity in sesame (*Sesamum indicum* L.). Plant Omics. 2010; 3(3): 85-91.

[15] Prakash V, Nandi PK. Association-dissociation behavior of sesame alpha globulin in electrolyte solutions. Journal of Biological chemistry. 1975; 252: 240-243.

[16] Rangarajan N, Ambilly M. Phytochemical screening of *Sesamum indicum* seed extract. World journal of pharmacy and pharmaceutical sciences. 2012; 1: 1298-1308.

[17] Moazzami AA, Haese SL, Kamal-Eldin A. Lignan contents in sesame seeds and products. European Journal of Lipid Science and Technology. 2007; 109: 1022-1027.

(18] Borchani C, Besbes S, Blecker C, Attia H. Chemical characteristics and oxidative stability of sesame seed, sesame paste and olive oils. Journal of Agricultural Science and Technology. 2010; 12:585-596.

[19] Abdul-Hamid A, Luan YS. Functional properties of dietary fiber prepared from defatted rice bran. Food chemistry. 2000; 15-19.

[20] Vishwanath HS, Anilakumar KR, Harsha SN, FarhathKhanum A, Bawa S. In vitro antioxidant activity of *Sesamum indicum* seeds. Asian Journal of Pharmaceutical and Clinical Research. 2011; 0974-2441.

[21] Nagendran B, Kalyana S, Samir S. Phenolic compounds in plants and agri-industrial by-product: antioxidant activity, ocuurence and potential uses. Food Chemistry. 2006; 191-203.

[22] Ensminger AH, Ensminger MK. Food for Health: A Nutrition Encyclopaedia Clovis, California: Pegus Press; 1986.

[23] Elleuch M, Bedigian D, Zitoun A. Sesame (*Sesamum indicum* L.) seeds in food, nutrition and health. In V. R. Preedy, R. R. Watson, V. B. Patel (Editors), Nuts & Seeds in Health and Disease Prevention (1st ed.) (pp 1029-1036). London, Burlington, San Diego. 2011; 122:1029-1036.

[24] Özcan M, Harmankaya M, Endes Z. Mineral contents and some physico-chemical properties of some commercial sesame seeds used in halva (sweet) production. International Journal of Farming and Allied Sciences IJFAS Journal. 2013; 115-119.
[25] Fallon S, Enig MG. Nourishing Traditions. The Cookbook that Challenges Politically Correct Nutrition and the Diet Dictocrats. Revised 2 Ed., 2001. p: 40-45.
[26] Nzikou JM, Matos L, Bouanga-Kalou G, Ndangui CB, Pambou-Tobi NPG, Kimbonguila A, Silou Th, Linder M, Desobry S. Chemical composition on the seeds and oil of sesame (*Sesamum indicum* L.) Grown in Congo Brazzaville. Advance Journal of Food Science and Technology. 2009; 1 (1): 6–11.
[27] Abdullahi Y, Adeniyi MO, Ihekwuemere CA. Countdown to senior secondary certificate exams in Agric. Science.Evans Brothers, Nigeria. 1991. p.150.
[28] Baydar H, Turgut K. Variation of certain characters and line selection for yield, oleic and linoleic acid in the Turkish sesame (*Sesamum indicum* L.) populations. Journal of Agriculture and Forestry. 1999; 23: 431 -441.
[29] Ogbonna PE, Ukaan SI. Chemical composition and oil quality of seeds of sesame accessions grown in the Nsukka plains of South Eastern Nigeria. African Journal of Agricultural Research. 2013; 8 (9): 797-803.
[30] Dim PE. Extraction and Characterization of Oil from Sesame Seed. Research Journal of Pharmaceutical, Biological and Chemical Sciences. 2013; 4 (2): 752-757.
[31] Weiss EA. Sesame. In: "Oilseed Crops". Longman Inc., New York. 1983.p. 282- 340.
[32] Abayeh OJ, Aina EA, Okuonghae CO. Oil content and oil quality characteristics of some Nigerian oil seeds. Journal of Pure Applied Sciences. 1998; 1:17-23.
[33] Gharby S, Harhar H, Guillaume D, Roudani A, Boulbaroud S, Ibrahimi M, Ahmad M, Sultana S, Ben Hadda T, Chafchaouni-Moussaoui I, Charrouf Z. Chemical Investigation of *Nigella sativa* L. Seed Oil Produced in Morocco. Journal of the Saudi Society of Agricultural Sciences. 2015;14(2) : 172-177.
[34] Lee J, Kim M, Choe E. Study on the changes of tocopherols and lignans and the oxidative properties of roasted sesame oil during manufacturing and storage. Korean Journal of Food Science and Technology. 2008; 40: 15–20.
[35] Guille´n MD, Ruiz A. Formation of hydroperoxy and hydroxyalkenals during thermal oxidative degradation of sesame oil monitored by proton NMR. Journal of Lipid Science and Technology. 2004; 106: 680-687.
[36] Abdalla I, khaddor M, Boussab A, El Garrouj D, Souhial B. Physical and Chemical Characteristics of Olive Oils from Cooperatives for Olive Growers in the North of Morocco. International Journal of Basic & Applied Sciences. 2014; 14(2):4-11.
[37] Gharby S, Harhar H, Guillaume D, Haddad A, Matthäus B, Charrouf Z. Oxidative stability of edible argan oil: A two year study. Food Science and Technology. 2011; 44(1):1-8.
[38] Markovic VV, Bastic LV. Characteristics of Pumpkin Seed Oil. Journal of American Oil Chemist Society. 1975; 53: 42-44.
[39] Ünal MK, Yalçin H. Proximate composition of Turkish sesame seeds and characterization of their oils. Grasas y Aceites. 2008; 59: 23-26.
[40] Hassan MAM. Studies on Egyptian sesame seeds (*Sesamum indicum* L.) and its products 1–physicochemical analysis and phenolic acids of roasted Egyptian sesame seeds (*Sesamum indicum* L.). World Journal of Dairy & Food Sciences. 2012; 7 (2): 195-201.
[41] Champolivier L, Merrien A. Evolution of the oil content and its fatty acid composition in two varieties of sunflower (oleic or not) under the effect of different temperatures during the maturation of the seeds. Oilseeds and fats, Crops and Lipids. 1996 ; 3(2) : 140-4.
[42] Lajara JR, Diaz U, Diaz Quidiello R. Definitive influence of location and climatic conditions on the fatty acid composition of sunflower seed oil. Journal of the American Oil Chemist's Society. 1990 ; 67: 618-623.
[43] Harwood JL. Fatty acids metabolism. Annual Review of Plant Physiology and Plant Molecular Biology. 1988; 39, 101-138.
[44] Yermanos DM, Saleeb W, Hemstree S, Huszar CK. Oil content and composition of seed in world collection of sesame introductions. Journal of the American Oil Chemist's Society. 1972; 49(1): 20-23.
[45] Uzun B , Arslan C , Furat S. Variation in fatty acid compositions, oil content and oil yield in a germplasm collection of sesame (*Sesamum indicum* L.). Journal of the American Oil Chemist's Society. 2008; 85:1135-1142.
[46] Kris-Etherton E. Bioactive compounds in foods: Their role in prevention on 71-cardiovascular disease and cancer. American Journal of Medicine. 2006; 113: 11-88.
[47] Mensink RP. Effects of stearic acid on plasma lipid and lipoproteins in humans. Lipids. 2005; 40(12):1201-1205.
[48] Liu Q, Singh SP, Green AG. High-oleic and high-stearic cottonseed oils: nutritionally improved cooking oils developed using gene silencing.The Journal of the American College of Nutrition. 2002; 21: 205-211.
[49] Aleksander S, Malgorzata N, Elenora L. The content and antioxidant activity of phenolic compounds in cold pressed plant oils. Journal of Food Lipids. 2008; 15: 137- 149.
[50] Tamara DP, Etelka BD, Aleksandar AT, Ranko RS. Influence of impurities and hull content in material for pressing on sensory quality cold –pressed sunflower oil. BIBLID: 2010; 41: 69- 76.
[51] Herchi W, Kallel H, Boukhchina S. Physicochemical properties and antioxidant activity of Tunisian date palm (*Phoenix dactylifera* L.) oil as affected by different extraction methods. Food Science andTechnology. 2014; 34(3): 464-470.
[52] Mansouri F, Ben moumen A, Lopez G, Fauconnier M, Sindic M, Serghini-Caid H, Elamrani A. Preliminary Characterization of monovarietal virgin olive oils produced in eastern area of Morocco. Inside Food Symposium. 2013; 9-12.
[53] El-Adawy TA, Mansour EH. Nutritional and physicochemical evaluations of tahina (sesame butter) prepared from heat-treated sesame seeds. Journal of Science of Food and Agriculture. 2000 ; 80: 2005-2011.
[54] Jannat B, Oveisi MR, Sadeghi N, Hajimahmoodi M, Behzad M, Choopankari E, Behfar AA. Effects of roasting temperature and time on healthy nutraceuticals of antioxidants and total phenolic content in iranian sesame seeds (*sesamum indicum* l.). Iranian Journal of Environmental Health Science & Engineering. 2010; 7: 97-102.
[55] Sadeghi N, Oveisi MR, Hajimahmoodi M, Jannat B, Mazaheri M, Mansouri S. The content of sesamol in Iranian sesame seeds. Iranian Journal of Pharmaceutical Research. 2009; 8: 101-105.
[56] Jeong SM, Kim SY, Kim DR, Nam KC, Ahn DU, Lee SC. Effect of seed roasting conditions on the antioxidant activity of defatted sesame meal extracts. Journal of Food Sciences. 2004; 69(5): 377-381.

[57] Mohamed HMA, Awatif I I. The Use of Sesame Oil Unsaponifiable Matter as a Natural Antioxidant. Food Chemistry. 1998; 62:269-276.
[58] Budowski P, Connor RT, Field ET. Sesame Oil. IV. Determination of Free and Bound Sesamol. Journal of the American Oil Chemist's Society. 1950; 27: 307-310.
[59] Lee SW, Jeung MK, Park MH, Lee SY, Lee JH. Effects of roasting conditions of sesame seeds on the oxidative stability of pressed oil during thermal oxidation. Food chemistry. 2010; 118: 681–685.
[60] Kajimoto G, Kanomi Y, Kawakami H, Hamatani M. Effects of Antioxidants on the Thermal Oxidation of Oils and Decomposition of Tocopherol in Vegetable Oils. Journal of Japan Society of Nutrition and Food Sciences. 1992; 45: 291-295.

Nutritional and functional properties of protein concentrate and protein isolates of foods

A. Cruz Solorio[1], **M. Garín Aguilar**[2] and **G. Valencia del Toro**[1]

[1] Laboratorio de Cultivos Celulares de la Sección de Estudios de Posgrado e Investigación, UPIBI, Instituto Politécnico Nacional, Barrio La Laguna SN. Ciudad de México, México

[2] Laboratorio de Farmacobiología de la Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México. Av de los Barrios No.1, Los Reyes Iztacala, Tlalnepantla, CP 54090. Edo. de México, México

Keywords: Protein isolates; Protein concentrate; functional properties; bioactive peptides

1. Introducction

In recent years, scientific evidence has increased about health benefits from proteins and bioactive peptides derived from food [1]. Protein and isolated extracts from flour have been obtained, reaching a final product with 80-90% of proteins [2]. For protein extraction, different techniques have been developed among them micellization, ultrafiltration, acidic and alkaline aqueous extraction followed by isoelectric precipitation [3]. Due to low solubility in protein sources, the obtainment of protein hydrolysates and even bioactive peptides has enhanced [4].

The protein concentrates of different food products have been reported considering species like rice (75.5%) [5], amaranth (77.8 %) [6], mushroom (48.56-49.94%) [7]; for bean protein isolates of 71.9 to 75.6 % [8], chickpea (85.40 %), lentil (81.90 %), pea (88.76 %) and soy (87.58 %) [9]. On the other hand, protein sources have been added in food elaboration/production due to its physicochemical properties like water and oil absorption capacities, gelification, and foaming and emulsifying properties, which affects food protein behavior and influence in quality and organoleptic characteristics from food system [10].

The present review provides information about nutritional composition and functional properties from different protein sources from some food. Likewise, extraction process for protein concentrates, protein isolates, protein hydrolysates and bioactive peptides. The section for existing applications of protein sources is also included.

2. Protein concentrate and isolates

In recent years the health benefits of bioactive proteins and peptides derived from some foods have been scientifically demonstrated [1]. Protein concentrates and isolates from the flour of different foods have been obtained, which decrease the non-protein components in order to obtain a final product with high protein content [2]. Depending on the protein concentration on a dry basis, they are named protein concentrates, with maximum values of 65% or protein isolates up to 90% [11]. However, in certain cases they may present a low solubility or allergenicity [4].

Protein concentrates are obtained when non-protein components such as carbohydrates, soluble minerals, antinutritional factors and some low molecular weight nitrogenous compounds are eliminated from food, and removed using aqueous-alcoholic solutions (ethanol, 1-butanol, isopropyl alcohol, etc.), acidic or basic solutions. In order to obtain protein isolates, proteins are solubilized in aqueous media by adjusting pH with sodium hydroxide [12].

3. Processing protein concentrate and protein isolate

Protein extraction and isolation involves solubilization and precipitation techniques based on their physicochemical properties [11]. One of the most used techniques in protein extraction is aqueous-alkaline extraction followed by precipitation, in which the proteins are solubilized at elevated alkaline pH and precipitate at pH near to their isoelectric point (~pH 4-5) [3]. Protein extractions of *Lupinus campestri* seeds at pH 9 (30 min, room temperature) have been reported followed by precipitation at pH 4.5, obtaining protein isolates with a 93.2% protein content [13], protein concentrates from mucuna seeds from degreased flour with an organic solvent in a 1:10 w/v ratio were obtained, adjusted pH to 8 for proteins solubilization and precipitated at pH 4, the protein percentage in this concentrate was 78.3% [14]. Likewise, Protein concentrates were also obtained from cowpea seed degreased flour by bringing the pH of the aqueous solution (1:3 w/v) to 9 and then precipitating the proteins at pH 4.33 [15]. Other compounds such as sodium hexametaphosphate (HMP), carboxy methyl cellulose (CMC), ammonium sulfate [16], methanol [17] etc. can be used.

In order to obtain protein concentrates from rice bran, proteins are solubilized at pH 9 and subsequently precipitated at pH 4.5. The obtained concentrate presents of 52.46 to 58.92% protein content [18]. Butt and Batool [19] prepared protein isolates from legume seeds using alkaline conditions at pH 9.5, the obtained precipitate was suspended in water

(1:5 w/v) and centrifuged, the supernatant of the latter process was combined with the first supernatant and the pH adjusted to 4.5, centrifuged, neutralized and lyophilized, obtaining protein isolates from pigeon pea, cowpea, mung bean and pea with values of 82.95%, 89.25%, 85.46%, and 83.61%, respectively. Also, to obtain protein concentrates from the baru nuts defatted flour solution (1:20 w/v), only alkaline extraction at pH 10 was used obtaining a 93% protein content [20]. Although the method of obtaining protein concentrates by alkaline extraction followed by acid precipitation is a traditional method for extracting proteins from plant species, it presents disadvantages due to protein denaturalization comprising the high concentrations of alkaline solutions. Maillard reactions are also likely to cause intense brown color in the protein concentrates and low nutritional value of the protein [21].

Another method to obtain protein concentrates is acid extraction, its extraction principle is similar to alkaline extraction except that it is carried out under acidic conditions (pH ~ 4), it has been observed that proteins solubility is high in very acidic conditions, so this physicochemical property is used to dissolve proteins at low pH and then carry out precipitation by adjusting the pH value of the isoelectric point of proteins [3]. Following this method Aremu et al. [22] obtained cashew nut protein concentrates with protein values of 69.6% using an aqueous solution of cashew nut degreased flour in 1:10 w/v concentration and adjusting pH with 0.1M HCl. On the other hand, acidic extractions have been reported at pH 2, followed by isoelectric precipitations at pH 4.5, obtaining soy protein isolates [23].

On the other hand, the micellization process (salt extraction) has also been used, which is based on the "salting-in" and "salting out" of proteins. In this process, after protein extraction with a salt solution inducing protein precipitation, the precipitate is recovered by centrifugation or filtration, followed by drying [3]. Sodium sulfite (Na_2SO_3) was used to solubilize proteins (pH 10.5, 4 ° C, 1 h) and then acidify the solution to the isoelectric point of proteins (pH 4) to obtain protein isolates with 92.4% protein content [24]. In addition, it has been reported that *Lupinus campestri* protein isolates were obtained with 95.7% protein extracted from degreased flour with 0.5 M sodium chloride and pH 7 [13].

Recently, membrane technology has been successful in recovering solubilized proteins. The use of ultrafiltration and diafiltration processes has allowed obtaining protein concentrates or isolates presenting low concentrations of soluble solids with low molecular weight, such as phytates, polyphenols and glucosinolates [11]. Protein concentrates were obtained from clarified lucerne juice with a 10 kDa ultrafiltration membrane at 242 kPa pressure and 16.7 rev/s speed, however the protein content was 35% lower than the obtained under acidic and neutral conditions that was 72% and 63%, respectively [25]. However, studies reporting 98.8% protein contents in concentrates obtained from rape seed acid supernatants, using 10 kDa ultrafiltration membranes, compared to the alkaline precipitation method in which concentrates were obtained with 70.8% [26].

Likewise, proteins based on their solubility have been consecutively extracted by the Osborne Method, for example, degreased rice flour is extracted with distilled water for 4 h at room temperature and then centrifuged to obtain the "albumen" fraction (supernatant). The obtained residue was extracted with 0.1 M NaOH solution for one hour to obtain the "glutelin" rich fraction. The latter extracted residue is combined with 70% ethanol to extract prolamins. Each albumin, globulin and glutelin fraction was precipitated according to its isoelectric point, ie 4.1, 4.3 and 4.8, respectively. A higher amount of glutelins (95.63%) was obtained, followed by prolamins (92%), globulins (90.41%) and to a lesser extent albumins (78.14%) [27].

Other factor that is important consider is the ionic strength effect on protein content, for example, protein isolates from sesame seeds were obtained by extraction with water (pH 7, 35 ° C) and in the presence of three NaCl concentrations (0.2, 0.6 and 1.0 M), followed by acid precipitation at pH 4.5 (25 ° C), dialysate and freeze-dried. At concentrations of 0 and 0.2 M NaCl the protein percentage was 100%, which decreases to 93.2% and 94.6% with concentrations of 0.6 and 1.0 M, respectively [28]. On the other hand, Elsohaimy et al. [29] indicated the positive effect of the agitation period during the extraction of quinoa proteins, which increases while agitation increases, presenting a maximum of protein extractability in 120 min.

It is important to indicate that during the process of obtaining protein isolates and concentrates, partial protein denaturalization can be caused by the drying process, since an insoluble protein aggregation is present irreversibly. The most commonly used methods are spray-dried, freeze-dried and vacuum-dried. However, in a research work it has been indicated that drying lentil protein isolates by the three aforementioned methods does not alter the physicochemical characteristics and their nutritional composition, no significant differences in protein content being found in a range of protein concentration from 90.2% to 91.9%, but there was an effect on the coloration of the protein isolate [30].

4. Nutritional value of concentrate and protein isolate

Protein characterization from isolates and concentrates is of great importance. Some of the characteristics that should be considered are: surface hydrophobicity, solubility, and electrophoretic patterns that indicate both the molecular weights of proteins and possible cross-linkings. Not forgetting the thermal properties, which are useful to know the protein changes during food cooking process [31]. In addition, nutritional quality and functional properties are also characteristics that must be considered, for their application in functional foods or nutraceuticals development [32].

Protein concentrates or isolates recovered from different vegetable or animal flours have a high protein content compared to the original flours, altering the moisture, fat, ashes, fiber, and carbohydrates contents (Table 1). For example, Nassar [33] reported a protein increase from 13.62% to 62.41% and decreases in other components from

protein concentrates of prickly pear seed, indicating that carbohydrates and fats in flour are removed at a great proportion during the protein concentrate process. In contrast, higher fat content (5.84%) was found in protein concentrates from cowpea flour (1.80%), this result is due to high fat content in protein concentrates or seed isolates of legumes caused by the binding between protein and lipid as a result of lipid emulsification by proteins [15].

Table 1 Nutritional composition of protein concentrate and protein isolate.

	Sample	Moisture	Protein	Fat	Ash	Fibre	Carbohydrate
Protein concentrate							
Prickly pear seed [33]	Flour	9.03	13.62	10.43	6.47	9.23	51.11
	PC	7.16	62.41	3.57	5.31	5.29	15.79
Pleurotus tuberregium [45]	Flour	12.5	17.6	5.95	6.4	5.80	51.8
	PC	6.35	5.24	1.50	40.4	4.26	42.2
Bambara beans [46]	Flour	6.45	24.78	5.9	3.88	5.35	52.94
	PC	8.92	70.85	13.15	3.86	1.82	11.25
Pleurotus ostreatus [7]	Flour						
	PCM	7.93	24.25	1.97	7.94	9.29	56.55
	POS	7.64	25.78	2.00	7.96	8.6000	55.65
	PCMXPOS	7.62	26.81	1.92	8.01	8.64	54.62
	PC						
	PCM	7.6	49.85	5.96	7.50	-	36.69
	POS	7.39	49.94	6.11	7.59		36.36
	PCMXPOS	7.22	48.56	5.66	7.59		38.19
Protein isolate							
Chickpea [47]	Flour	8.1	24.7	1.5	3.7	18.8	51.3
	PI						
	Isolate-A	3.3	78.0	3.5	2.9	3.8	11.8
	Isolate-B	5.5	88.1	1.1	4.3	3.2	3.3
Lupin [48]	Flour	7.9	33.8	13.6	2.1	39.9	ND
	PI						
	Isolate A	3.4	87.4	3.2	0.7	4.0	ND
	Isolate B	9.4	83.9	1.0	0.3	4.8	
Legumes [49]	Flour						
	Lathyrus clymenum	11.40	21.25	1.99	2.58	41,.10	19.05
	Lathyrus annuus	10.90	19.95	1.93	2.20	41.0	20.9
	PI						
	Lathyrus clymenum	4.64	82.40	1.16	0.26	9.18	Tr
	Lathyrus annuus	4.78	81.07	1.09	0.20	7.71	

Note: PC: protein concentrate; PI: protein isolate

Gueguen (1983) [34] mentioned that lipids are free in flours but they are trapped by proteins during the process of obtaining protein concentrates. In other investigations, protein concentrates of rice bran showed protein content in dry basis between 52.46% and 58.92% [18], 79% in protein concentrates of soybean [35], 36.42% in amaranth leaf protein concentrates [36], whereas for amaranth seeds the percentage of proteins is between 73.6% and 77.8% [6], a 75.5% in rice protein concentrates [5], and even the protein content in mesquite concentrates has been doubled to 67.9% from flour which has only 33.8% [37]. There are reports of bean protein isolates presenting 83.96-89.25% of proteins, three times more than bean flours (22.36-28.5%), considering that ashes, carbohydrate and lipid content are reduced in these isolates [38], protein content has also increased to 75.8% and 83.4% in amaranth seeds protein isolates [39]. Also, to 89.9%-94.4% was reported for protein isolates from chickpea defatted flour which contained 20.6 and 26.7% of proteins [40], for rice flour protein isolates, protein content between 79% and 91.1% was obtained [41], in beach pea isolates, the reported protein content was 85.1% and 86.6% [42].

The physicochemical processes used in the different obtaining methods for isolates and proteinaceous compounds such as alkaline extraction-isoelectric precipitation, micellization, acid extraction, as well as the use of high temperatures and/or organic solvents to degrease the sample before protein extraction can cause protein denaturalization, affecting both the solubility and yield of protein concentrates and isolates [43]. 3 Valor nutricional/Pedroche et al 2004 AP.pdf. Regarding to yield, Wani et al. [44] reported 35.15% and 38.27% for protein isolates from watermelon seed flour, whereas in protein concentrates the yield was lower than 25.21 % and 27.41%, also indicating that dry basis protein content was from 71.38 to 83.79%. In another study, a protein content of 75.8% in amaranth protein isolates was reported on extraction at pH 8.5 and precipitation at pH 5.0, on the other hand, by increasing the extraction pH to 9.0 and lowering the pH of precipitation to 4.5, the protein content increased to 83.4% [39].

It is important to mention that the objective of removing undesirable components during the process of obtaining protein products is essential to improve their nutritional quality and to know their potential as a functional ingredient [39].

5. Functional properties of protein concentrate and protein isolate

Functional properties are physicochemical properties of proteins and affect their organoleptic characteristics and quality, interfering in the behavior and appearance of food from its preparation to its storage [50, 51]. Proteins can be used as structural stabilizers in foods, such as emulsifiers or foaming agents and even for forms or stabilizing gels [52]. Therefore the functionality of a protein will allow us to know the type of product in which it would be used and plays an important role in consumer acceptance [53].

5.1 Solubility

The amino acid that conforms the proteins presents a prevailing charge at different pHs which determines their solubility. Figure 1 shows the possible configurations of the functional groups present in the amino acids of a protein, in the region of the isoelectric point predominates a molecule called "ion zwitterrion" (I), where there is a balance in the positive and negative charges minimizing the electrostatic repulsion and as a consequence, a reduction in protein solubility. With acidic pH values lower than the isoelectric point, the cation III predominates, whereas in an alkaline medium the anion II takes precedence. In both cases, there is an improvement in electrostatic repulsion and higher solubility at pH 2 and pH 11, respectively [54].

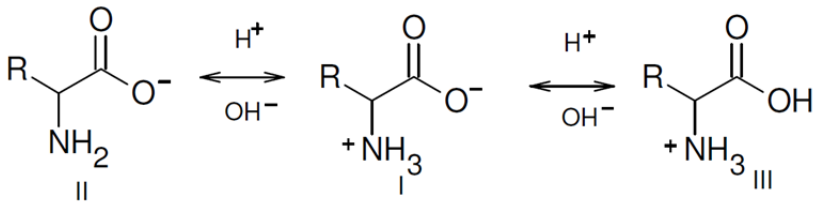


Fig. 1 Prevalent charge on proteins at different pH.

For the obtaining process for oat bran protein concentrates in acid medium, solubility percentages of smaller proteins (4.7 to 7.3%, with pH 5) have been reported compared to those obtained in alkaline medium (pH 9) reaching up to 89.3% in protein solubility [55]. In another study, under acidic conditions at pH 2, the protein concentrates of foxtail millet and soybeans had a solubility of 40%, which decreased by about 20% when the values were pH 4 and pH 5, however at pH 6, solubility increased between 50% and 80% [56]. Similarly, for soybean and faba beans protein isolates, the maximum solubility was pH 8 and pH 9, and low solubility between pH 4 and 6, because the proteins of most legumes precipitate at pH 4 [57].

5.2 Water and oil absorption capacity

Water absorption capacity (WAC) and oil absorption capacity (OAC) are functional properties of food proteins that are important in the development of new products, particularly for flavor fixation, as well as in the development of oxidative rancidity during storage [58]. In particular WAC from a protein isolate determines the interaction degree for water and protein solubility, influencing surface tension, binding energy, temperature, pH, ionic force, vapor pressure, among other physicochemical factors [59, 60]. In the case of protein isolates, values for WAC between 97% and 163% for legume seeds has been found, where pigeon pea isolates showed lower WAC, followed by cowpea, and peas and mung bean with higher WAC [61]. Ogundele et al. [62], indicated that protein isolates from melon seeds showed high WAC (2.67-3.5 g/g) due to their high protein content and consequently a high hydrolysis capacity, on the other hand OAC of these protein isolates (2.24-3.14 g/g) was higher in comparison to flour (0.84-1.4 g/g). Similar behavior was found for cashew nut protein isolates, which presented higher OAC (4.42 ml oil/g) than protein concentrate (3.32 mL oil/g), but both products showed higher values than flour (2.05 mL oil/g) [63]. Proteins containing higher non-polar functional groups are more hydrophobic and have an important role in oil absorption because they show greater binding to proteins, such binding is achieved between the lipid chains with the non-polar amino acid side chains [64], furthermore the conformational structure that proteins adopt is also important [65], so hydrophilic and hydrophobic proteins behavior affects OAC [66]; the increase of WAC also causes physical retention by capillarity in the new structures by aggregation of formed proteins [67].

5.3 Gelation

The critical factor for gel formation is protein concentration, not requiring a high solubility to form the gel [67]. Other important factors are electrostatic and hydrophobic interactions, hydrogen and disulfide bonds, and protein aggregation. In addition, the degreasing process favors gels formation with low lipid content and high protein content and as a consequence, the decrease in the least gelation concentration (LGC) [68]. If the LGC is lower, the gelation capacity of the protein ingredient is higher [69]. For example, cashew nut degreased flour showed 6.5% of LGC, 10.0% for protein concentrates and 13.5% for protein isolates [63].

In contrast, flour and protein fractions obtained from rice had a LGC of 4% and 5%, respectively [27]. Variations in gelling properties are due to the proportion of different constituents such as proteins, lipids and carbohydrates in flours [70]. In addition, the gel properties are influenced by several factors, such as pH, their nature, electrolytes and proteins concentration [71].

5.4 Foam properties

Foam is a colloidal system formed by tiny air bubbles dispersed in an aqueous continuous phase called lamella. Bubbles formation and stability is achieved by incorporating an agent capable of reducing the surface tension between the phases. Proteins are good foam stabilizers because they act at the air:water interface, where the hydrophobic residues of the amino acids present in the polypeptide chain are oriented towards the interior of the bubble and the hydrophilic part oriented toward the aqueous phase [72]. A good foaming capacity has been linked to proteins flexibility with reduced surface tension and with a high protein concentration [73]. For example, Segura-Campos et al. [74] evaluated the foaming capacity respecting to pH of bean protein concentrates, the lowest foam capacity was at pH 4 and higher under alkaline conditions (pH 8), indicating that it may be due to an increase in protein net charge which weakens hydrophobic interactions by improving their flexibility and enhancing foaming. Foam stability decreases in neutral pH regarding to time (0.5 min, 5 min, 30 min, and 120 min), but increases in acidic and alkaline pH.

In other investigations, protein isolates from cashew nut shell showed minimum foaming capacity at pH 3 (28.65%) whereas in alkaline conditions it was 83.45-86.51%, and for acidic conditions at pH 2 it increased to 60.13%. A similar behavior was observed for its stability, at pH 3 it was 33.89% increasing to 83.75% at pH 11, attributed to proteins solubility increases when pH is above the isoelectric point [75]. Similar results have been found in protein products of peanut, at a time of 0 min, flour and protein concentrates presented low foaming capacity corresponding to 28% for flour and 26.5% for concentrates, in contrast for protein isolates of 50%. After 30 and 60 min it does not decrease significantly in each product, which indicates that they are good foam stabilizers, and that higher protein concentration, foaming capacity and foam stability also increases [76]. For a protein to be a good foaming agent it must meet two basic requirements: 1) the ability to absorb at the water-oil interface and 2) the ability to undergo conformational changes at the interface [77].

On the other hand, it has been reported that the protein concentrate of edible fungus *Pleurotus tuber-regium* presented greater foam stability compared to flour [46]. In addition, foaming capacities depend on proteins and other components such as carbohydrates, which are present in flours [78]. Also, the ability of a protein to form or stabilize foam will depend on the type of protein and the degree of denaturalization, the presence of calcium ions, pH, temperature and whipping methods [79].

5.5 Emulsion properties

According to Mena-Casanova and Totosaus [80], the emulsion capacity (EC) measures the ability of soluble proteins to migrate to the water-oil interface, so it must be considered that solubility and conformation of proteins are affected by environmental conditions such as ionic strength and pH. The determination of EC depends on the water-oil interface and requires a large amount of emulsifier to stabilize the emulsion and the droplets size produced during stirring. Also, other parameters have to be taken into account to measure the ability of the protein to form an emulsion, among them is the emulsion activity index (EAI) and the emulsion stability index (ESI). EAI is measured as the interfacial area stabilized per mass unit of protein based on the emulsion turbidity and the change in turbidity of that diluted emulsion measures the ESI per unit time [81, 82].

The emulsion activity indexes of protein isolates from sesame seeds were 16.8 m^2/g, higher than those obtained in protein isolates of soybean (12.2 m^2/g). However, for sesame protein isolates this activity was improved at pH 5 (20.7 m^2/g) and decreased at pH 2 (13.0 m^2/g), these results confirm that the emulsifying properties are pH dependent [83]. Another study in peanut protein isolates showed higher emulsion stability indexes at alkaline pH (7 and 9) and at acidic pH (pH 3) [84]. Similar behavior was observed in flours, concentrates and walnut protein isolates, where the emulsion activity was higher at pH 2 and 12, and decreased at the isoelectric point of proteins (pH 4.5), but at all pH the flour presented the higher values compared to concentrate and protein isolate [85].

On the other hand, surface protein concentration between flour and coriander protein concentrates has been determined, the latter had low protein concentrations per surface area (mg/m^2) compared to flour, and this is attributed to the fact that flour contains non-protein components, which reduce electrostatic repulsions between proteins [86]. In conclusion, the difference in total protein composition and non-protein contribute to the emulsifying properties of protein products, that is, the proteins decrease surface tension and promote electrostatic repulsions between them, whereas some carbohydrates increase the system viscosity to stabilize the emulsion [87]. A protein capacity to improve the formation and stabilization of emulsions is important for food industry applications such as cakes, coffee bleaches and frozen desserts [88].

6. Hydrolysates and Peptides bioactives

As previously mentioned, protein isolates have limitations in the food industry, which is why protein hydrolysis processes have been carried out, aiming at to the peptide bond breakdown and consequently the generation of smaller peptides or amino acids release. To achieve the bond rupture, chemical methods with the addition of acids or bases, or biological with enzymes application are necessary [2]. This latter method has an advantage in comparison to the others, since enzymes are selective and specific for the breaking of a certain bond, it is carried out in moderate temperature and pH conditions, and maintains the nutritive value because there is no degradation of separate components, while the amino acids arginine and cysteine are destroyed in alkaline and acidic hydrolysis and even tryptophan is eliminated [89].

When obtaining protein hydrolysates, certain functional characteristics such as decrease in viscosity, greater agitation capacity, dispersion and increase in solubility are favored, advantages those are desirable to be incorporated into food products. In addition, the fundamental property of hydrolysates is the degree of hydrolysis, which determines its possible use, is defined as the percentage of breakage of peptide bonds in relation to the original protein and is grouped in: hydrolysates with low hydrolysis degree (between 1% and 10%) for the improvement of functional properties; Hydrolysates with a variable hydrolysis degree to be used as flavorings, and finally, hydrolysates with hydrolysis degree greater than 10%, in specialized feed [90].

Through enzymatic hydrolysis, peptides can be generated, which are specific protein fragments and have the characteristic of being inactive within the sequence of the original protein from where they were obtained and once released they present bioactivity. The molecular weight of peptides is less than 6 kDa and formed from 2 to 20 amino acids [91]. The potential of bioactive peptides from animal or vegetable origin makes it attractive as an ingredient in food and pharmaceutical industries. Bioactivity has been found in peptides obtained from the hydrolysis of milk, egg and other proteins [92].

In addition, several biological activities have been shown in peptides and hydrolysates, such as anti-inflammatory activity [93], antioxidant [94], antihypertensive [95], antibacterial [96, 97, 98], inhibitory activity of angiotensin-I converting enzyme (ACE I) [99, 100], and immunomodulatory [101].

In conclusion, protein concentrates and isolates from plant, animals, and even fungi represent a good source of high quality proteins and important functional properties making them attractive for food processing. Also the production of hydrolysates and peptides with biological activities are important for the food and pharmaceutical industries.

Acknowledgements The support by SIP-IPN 20170419 is gratefully acknowledged.

References

[1] Möller NP, Bioactive peptides and proteins from foods: indication for health effects. European Journal of Nutrition, 2008; 47(4):171-182.
[2] Vioque J, Obtención y aplicaciones de concentrados y aislados proteicos. Grasas y aceites, 2001;52(2):127-131.
[3] Boye J, Zare F, Pletch A. Pulse proteins: processing, characterization, functional properties and applications in food and feed. Food Research International, 2010; 43(2):414-431.
[4] Vioque J, Pedroche J, Yust M, Lqari M, Megías C, Girón-Calle J, Alaiz M, Millán F. Bioactive peptides in storage plant proteins. Brazilian Journal of Food Technology. 2006; 99-102.
[5] Palmegiano GB. Rice protein concentrate meal as a potential ingredient in practical diets for rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 2006; 258(1):357-367.
[6] Bejarano-Luján DL, Netto FM. Effect of alternative processes on the yield and physicochemical characterization of protein concentrates from *Amaranthus cruentus*. LWT-Food Science and Technology, 2010; 43(5):736-743.
[7] Cruz-Solorio A, Proximate composition of *Pleurotus* fruit body flour and protein concentrate. Journal of Chemical, Biological and Physical Sciences, 2014; 4(5):52-60.
[8] Morales-de León JC, Preparation and characterization of protein isolate from fresh and hardened beans (*Phaseolus vulgaris* L.). Journal of Food Science, 2007; 72(2):C96-C102.
[9] Karaca AC, Low N, Nickerson M. Emulsifying properties of chickpea, faba bean, lentil and pea proteins produced by isoelectric precipitation and salt extraction. Food Research International, 2011; 44(9):2742-2750.
[10] Messens W, Van Camp J, Huyghebaert A. The use of high pressure to modify the functionality of food proteins. Trends in Food Science & Technology, 1997; 8(4):107-112.
[11] Rodrigues IM, Coelho JF, Carvalho MGV. Isolation and valorisation of vegetable proteins from oilseed plants: Methods, limitations and potential. Journal of Food Engineering. 2012; 109(3):337-346.
[12] Moure A, Sineiro J, Domínguez H, Parajó JC. Functionality of oilseed protein products: a review. Food Research International. 2006; 39(9):945-963.
[13] Rodríguez-Ambriz SL, Martínez-Ayala AL, Millán F, Davila-Ortiz G. Composition and functional properties of Lupinus campestris protein isolates. Plant Foods for Human Nutrition (Formerly Qualitas Plantarum). 2005; 60(3):99-107.
[14] Adebowale KO, Lawal OS. Foaming, gelation and electrophoretic characteristics of mucuna bean (Mucuna pruriens) protein concentrates. Food Chemistry. 2003; 83(2):237-246.

[15] Olivera-Castillo L, Pereira-Pacheco F, Polanco-Lugo E, Olvera-Novoa M, Rivas-Burgos J, Grant G. Composition and bioactive factor content of cowpea (*Vigna unguiculata* L. Walp) raw meal and protein concentrate. Journal of the Science of Food and Agriculture. 2007; 87(1):112-119.
[16] Saetae D, Kleekayai T, Jayasena V, Suntornsuk W. Functional properties of protein isolate obtained from physic nut (*Jatropha curcas* L.) seed cake. Food Science and Biotechnology. 2001; 20(1):29-37.
[17] Ogunwolu SO, Henshaw FO, Mock HP, Santros A, Awonorin SO. Functional properties of protein concentrates and isolates produced from cashew (*Anacardium occidentale* L.) nut. Food Chemistry. 2009; 115(3):852-858.
[18] Chandi GK, Sogi DS. Functional properties of rice bran protein concentrates. Journal of Food Engineering. 2007; 79(2):592-597.
[19] Butt MS, Batool R. Nutritional and functional properties of some promising legumes protein isolates. Pakistan Journal of Nutrition. 2010; 9(4):373-379.
[20] Guimarães RDCA, Favaro SP, Viana ACA, Braga Neto JA, Neves VA, Honer MR. Study of the proteins in the defatted flour and protein concentrate of baru nuts (*Dipteryx alata* Vog). Food Science and Technology (Campinas). 2012; 32(3):464-470.
[21] Hou F, Ding W, Qu W, Oladejo AO, Xiong F, Zhang W, He R, Ma H. Alkali solution extraction of rice residue protein isolates: Influence of alkali concentration on protein functional, structural properties and lysinoalanine formation. Food Chemistry. 2017; 218:207-215.
[22] Aremu MO, Ogunlade I, Olonisakin A. Fatty acid and amino acid composition of protein concentrate from cashew nut (Anarcadium occidentale) grown in Nasarawa State, Nigeria. Pakistan Journal of Nutrition. 2007; 6(5):419-423.
[23] Abirached C, Medrano CA, Panizzolo LA, Moyna P, Añón MC. Estabilidad de espumas formuladas con proteínas de soja tratadas a pH ácido. Innotec Revista del Laboratorio Tecnológico del Uruguay. 2005; 5:58-62.
[24] Vioque J, Alaiz M, Girón-Calle J. Nutritional and functional properties of Vicia faba protein isolates and related fractions. Food Chemistry. 2012; 132(1):67-72.
[25] Lamsal BP, Koegel RG, Gunasekaran S. Some physicochemical and functional properties of alfalfa soluble leaf proteins. LWT-Food Science and Technology. 2007; 40(9):1520-1526.
[26] Yoshie-Stark Y, Wada Y, Wäsche A. Chemical composition, functional properties, and bioactivities of rapeseed protein isolates. Food Chemistry, 2008; 107(1):32-39.
[27] Agboola S, Ng D, Mills D. Characterization and functional properties of Australian rice protein isolates. Journal of Cereal Science. 2005; 41(3):283-290.
[28] Auchouri A, Nail V, Boye JI. Sesame protein isolate: Fractionation, secondary structure and functional properties. Food Research International. 2012; 46(1):360-369.
[29] Elsohaimy SA, Refaay TM, Zaytoun MAM. Physicochemical and functional properties of quinoa protein isolate. Annals of Agricultural Sciences. 2015; 60(2):297-305.
[30] Joshi M, Adhikari B, Aldred P, Panozzo JF, Kasapis S. Physicochemical and functional properties of lentil protein isolates prepared by different drying methods. Food Chemistry. 2011; 129(4):1513-1522.
[31] Horax R, Hettiarachchy N, Kannan A, Chen P. Protein extraction optimisation, characterisation, and functionalities of protein isolate from bitter melon (Momordica charantia) seed. Food chemistry. 2011; 124(2):545-550.
[32] Matak KE, Tahergorabi R, Jaczynski J. A review: Protein isolates recovered by isoelectric solubilization/precipitation processing from muscle food by-products as a component of nutraceutical foods. Food Research International. 2015, 77:697-703.
[33] Nassar AG. Chemical composition and functional properties of prickly pear (*Opuntia ficus* indica) seeds flour and protein concentrate. World Journal of Dairy and Food Sciences. 2008; 3(1):11-16
[34] Gueguen J. Legume seed protein extraction, processing, and end product characteristics. Plant Foods for Human Nutrition (Formerly Qualitas Plantarum). 1993; 32(3):267-303.
[35] Chinma CE, Ariahu CC, Abu JO Chemical composition, functional and pasting properties of cassava starch and soy protein concentrate blends. Journal of Food Science and Technology. 2013; 50(6):1179-1185.
[36] Ngugi CC, Oyoo-Okoth E, Manyala JO, Fitzsimmons K, Kimotho A. Characterization of the nutritional quality of amaranth leaf protein concentrates and suitability of fish meal replacement in Nile tilapia feeds. Aquaculture Reports. 2017; 5:62-69.
[37] Jaimes JDC, Restrepo DA, Acevedo D. Preparación y determinación de las propiedades funcionales del concentrado proteico de trupillo (*Prosopis juliflora*). Biotecnología en el Sector Agropecuario y Agroindustrial. 2014; 12(1).
[38] Rui X, Boye JI, Ribereau S, Simpson BK, Prasher SO. Comparative study of the composition and thermal properties of protein isolates prepared from nine *Phaseolus vulgaris* legume varieties. Food Research International. 2011; 44(8):2497-2504.
[39] Salcedo-Chávez B, Osuna-Castro JA, Guevara-Lara F, Domínguez-Domínguez J, Paredes-López, O. Optimization of the isoelectric precipitation method to obtain protein isolates from amaranth (*Amaranthus cruentus*) seeds. Journal of Agricultural and Food Chemistry. 2002; 50(22):6515-6520.
[40] Kaur M, Singh N. Characterization of protein isolates from different Indian chickpea (*Cicer arietinum* L.) cultivars. Food Chemistry. 2007; 102(1):366-374.
[41] Shih FF, Daigle KW. Preparation and characterization of rice protein isolates. Journal of the American Oil Chemists' Society. 2002; 77(8):885-889.
[42] Chavan UD, McKenzie DB, Shahidi F. Functional properties of protein isolates from beach pea (Lathyrus maritimus L.). Food chemistry. 2001; 74(2):177-187.
[43] Pedroche J, Yust MM, Lqari H, Girón-Calle J, Alaiz M, Vioque J, Millán F. Brassica carinata protein isolates: chemical composition, protein characterization and improvement of functional properties by protein hydrolysis. Food Chemistry. 2004; 88(3):337-346.
[44] Wani AA, Sogi DS, Singh P, Shivhare US. Characterization and functional properties of watermelon (*Citrullus lanatus*) seed protein isolates and salt assisted protein concentrates. Food Science and Biotechnology. 2011; 20(4):877-887.
[45] Alobo AP. Proximate composition and functional properties of *Pleurotus tuberregium* sclerotia flour and protein concentrate. Plant Foods for Human Nutrition (Formerly Qualitas Plantarum). 2003; 58(3):1-9.

[46] Mune MAM, Minka SR, Mbome IL, Etoa FX. Nutritional potential of Bambara bean protein concentrate. Pakistan Journal of Nutrition. 2011; 10(2):112-119.
[47] Sánchez-Vioque R, Clemente A, Vioque J, Bautista J, Millán F. Protein isolates from chickpea (*Cicer arietinum* L.): chemical composition, functional properties and protein characterization. Food Chemistry. 1999; 64(2):237-243.
[48] Lqari H, Vioque J, Pedroche J, Millán F. Lupinus angustifolius protein isolates: chemical composition, functional properties and protein characterization. Food Chemistry. 2002; 76(3):349-356.
[49] Pastor-Cavada E, Juan R, Pastor JE, Alaiz M, Vioque J. Protein isolates from two Mediterranean legumes: *Lathyrus clymenum* and *Lathyrus annuus*. Chemical composition, functional properties and protein characterisation. Food chemistry. 2010; 122(3):533-538.
[50] Messens W, Van Camp J, Huyghebaert A. The use of high pressure to modify the functionality of food proteins. Trends in Food Science & Technology. 1997; 8(4):107-112.
[51] Ramírez A, Pacheco de Delahaye E. Propiedades funcionales de harinas altas en fibra dietética obtenidas de piña, guayaba y guanábana. Interciencia. 2009; 34(4):293-298.
[52] Foegeding EA, Davis JP. Food protein functionality: a comprehensive approach. Food Hydrocolloids. 2011; 25(8):1853-1864.
[53] Chel GLA, Corso RL, Betanacur-Ancona DA. Estructura y propiedades funcionales de proteínas de leguminosas. Revista de la Universidad Autónoma de Yucatán. 2003; 34-43.
[54] Adebowale YA., Adeyemi IA, Oshodi AA. Functional and physicochemical properties of flours of six *Mucuna* species. African Journal of Biotechnology. 2005; 4(12).
[55] Guan X, Yao H, Chen Z, Shan L, Zhang M. Some functional properties of oat bran protein concentrate modified by trypsin. Food Chemistry. 2007; 101(1):163-170.
[56] Mohamed TK, Zhu K, Issoufou A, Fatmata T, Zhou H. Functionality, in vitro digestibility and physicochemical properties of two varieties of defatted foxtail millet protein concentrates. International Journal of Molecular Sciences. 2009; 10(12):5224-5238.
[57] Fernández-Quintela A, Macarulla MT, Del Barrio AS, Martínez JA. Composition and functional properties of protein isolates obtained from commercial legumes grown in northern Spain. Plant Foods for Human Nutrition (Formerly Qualitas Plantarum). 1997; 51(4):331-341.
[58] Siddiq M, Ravi R, Harte JB, Dolan KD. Physical and functional characteristics of selected dry bean (*Phaseolus vulgaris* L.) flours. LWT-Food Science and Technology. 2010; 43(2):232-237.
[59] Paredes-López O, Ondorica-Falomir C, Olivares-Vázquez M. Chickpea protein isolates: Physicochemical, functional and nutritional characterization. Journal Food Science. 1991; 56:726–729.
[60] Chavan UD, McKenzie DB, Shahidi F. Functional properties of protein isolates from beach pea (*Lathyrus maritimus* L.). Food Chemistry. 2001; 74(2):177-187.
[61] Butt MS, Batool R. Nutritional and functional properties of some promising legumes protein isolates. Pakistan Journal of Nutrition. 2010; 9(4):373-379.
[62] Ogundele JO, Oshodi AA, Sanni TA, Amoo IA. Protein isolates of gourd melon seeds and their functional properties. American Journalof Food and Nutrition. 2013; 3(4):176-181.
[63] Ogunwolu SO, Henshaw FO, Mock HP, Santros A, Awonorin SO. Functional properties of protein concentrates and isolates produced from cashew (*Anacardium occidentale* L.) nut. Food Chemistry. 2009; 115(3):852-858.
[64] Du SK, Jiang H, Yu X, Jane JL. Physicochemical and functional properties of whole legume flour. LWT-Food Science and Technology. 2014; 55(1):308-313.
[65] Kinsella J. Functional properties of soy proteins. Journal of the American Oil Chemist's Society. 1979; 56:242–249.
[66] Jitngarmkusol S, Hongsuwankul J, Tananuwong K. Chemical compositions, functional properties, and microstructure of defatted macadamia flours. Food Chemestry. 2008; 110:23–30
[67] Sangronis E. Propiedades funcionales de las harinas de leguminosas (*Phaseolus vulgaris* y *Cajan cajan*) germinadas. Interciencia. 2004; 29(2):80-85.
[68] Joshi AU, Liu C, Sathe SK. Functional properties of select seed flours. LWT-Food Science Technology. 2015; 60(1):325-331.
[69] Maninder K, Sandhu KS, Singh N. Comparative study of the functional, thermal and pasting properties of flours from different field pea (*Pisum sativum* L.) and pigeon pea (*Cajanus cajan* L.) cultivars. Food chemistry. 2007; 104(1):259-267.
[70] Nithiyanantham S, Siddhuraju P, Francis G. Proximate composition and functional properties of raw and processed Jatropha curcas L. Kernel meal. International Journal of Research in Pharmaceutical and Biomedical Sciences. 2013; 4(1):183-195.
[71] Alleoni ACC. Albumen protein and functional properties of gelation and foaming. Scientia Agricola. 2006; 63(3):291-298.
[72] Camacho DRB, Moreno MJ, García D, Medina C, Sidorovas A.Caracterización de un hidrolizado proteico enzimático obtenido del pez caribe colorado (*Pygocentrus cariba* Humboldt, 1821). Interciencia. 2007; 32(3):188-194.
[73] Chaparro S, Gil J and Aristizábal I. Effect of hydration and baking on the physical and functional properties of vitabosa flour (*Mucuna deeringiana*). VITAE Revista de la Facultad de Química Farmacéutica. 2011; 18:133-143.
[74] Segura-Campos MR, Cruz-Salas J, Chel-Guerrero L, Betancur-Ancona D. Chemical and Functional Properties of Hard-to-Cook Bean (*Phaseolus vulgaris*) Protein Concentrate. Food and Nutrition Sciences. 2014; 5(21):2081.
[75] Yuliana M, Truong CT, Huynh LH, Ho QP, Ju YH. Isolation and characterization of protein isolated from defatted cashew nut shell: Influence of pH and NaCl on solubility and functional properties. LWT-Food Science and Technology. 2014; 55(2):621-626.
[76] Wu H, Wang Q, Ma T, Ren J. Comparative studies on the functional properties of various protein concentrate preparations of peanut protein. Food Research International. 2009; 42(3):343-348.
[77] Kumar KS, Ganesan K, Selvaraj K, Rao PS. Studies on the functional properties of protein concentrate of *Kappaphycus alvarezii* (Doty) Doty–An edible seaweed. Food chemistry. 2014; 153:353-360.
[78] Sreerama YN, Sashikala VB, Pratape VM, Singh V. Nutrients and antinutrients in cowpea and horse gram flours in comparison to chickpea flour: Evaluation of their flour functionality. Food Chemistry. 2012; 131(2):462-468.

[79] Ahmedna M, Prinyawiwatkul W, Rao RM. Solubilized wheat protein isolate: functional properties and potential food applications. Journal of Agricultural and Food Chemistry. 1999; 47(4):1340-1345.
[80] Mena-Casanova E, Totosaus A. Improvement of emulsifying properties of milk proteins wit.h κ or λ carrageenan: effect of pH and ionic strength. International journal of food science & technology. 2011; 46(3)535-541.
[81] Pearce KN, Kinsella JE. Emulsifying properties of proteins: evaluation of a turbidimetric technique. Journal of Agricultural and Food Chemistry. 1978; 26(3):716-723.
[82] Kaushik P, Dowling K, McKnight S, Barrow CJ, Wang B, Adhikari B. Preparation, characterization and functional properties of flax seed protein isolate. Food Chemistry. 2016; 197:212-220.
[83] Achouri A, Nail V, Boye JI. Sesame protein isolate: Fractionation, secondary structure and functional properties. Food Research International. 2012; 46(1):360-369.
[84] Jamdar SN, Rajalakshmi V, Pednekar MD, Juan F, Yardi V, Sharma A. Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. Food Chemistry, 2010; 121(1):178-184.
[85] Mao X, Hua Y. Composition, structure and functional properties of protein concentrates and isolates produced from walnut (*Juglans regia* L.). International Journal of Molecular Sciences. 2012; 13(2):1561-1581.
[86] Aluko RE, McIntosh T, Reaney M. Comparative study of the emulsifying and foaming properties of. defatted coriander (*Coriandrum sativum*) seed flour and protein concentrate. Food Research International. 2001; 34(8)733-738.
[87] Wani IA, Sogi DS, Wani AA, Gill BS. Physico-chemical and functional properties of flours from Indian kidney bean (*Phaseolus vulgaris* L.) cultivars. LWT-Food Science and Technology. 2013; 53(1): 278-284.
[88] Chandra S, Samsher. Assessment of functional properties of different flours. African Journal of Agricultural Research. 2013; 8(38):4849-4852.
[89] Guadix A., Guadix EM, Páez-Dueñas MP, González-Tello P, Camacho F. Procesos tecnológicos y métodos de control en la hidrólisis de proteínas. Ars Pharmaceutica. 2000; 41(1):79-89.
[90] Benítez R, Ibarz A, Pagan J. Hidrolizados de proteína: procesos y aplicaciones. Acta Bioquímica Cclínica Latinoamericana. 2008; 42(2):227-236.
[91] Sarmadi BH, Ismail A. Antioxidative peptides from food proteins: a review. Peptides. 2010; 31(10):1949-1956.
[92] Hartmann R, Meisel H. Food-derived peptides with biological activity: from research to food applications. Current Opinion in Biotechnology. 2007; 18(2):163-169.
[93] Oseguera-Toledo ME, de Mejia EG, Dia VP, Amaya-Llano SL. Common bean (*Phaseolus vulgaris L.*) hydrolysates inhibit inflammation in LPS-induced macrophages through suppression of NF-κB pathways. Food Chemistry. 2011; 127(3):175-1185.
[94] He R, Girgih AT, Malomo SA, Ju X, Aluko RE. Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. Journal of Functional Foods. 2013; 5(1):219-227.
[95] Kawasaki T, Seki E, Osajima K, Yoshida M, Asada K, Matsui T, Osajima Y. Antihypertensive effect of valyl-tyrosine, a short chain peptide derived from sardine muscle hydrolyzate, on mild hypertensive subjects. Journal of human hypertension. 2000; 14(8):519.
[96] Demers-Mathieu V, Gauthier SF, Britten M, Fliss I, Robitaille G, Jean J. Antibacterial activity of peptides extracted from tryptic hydrolyzate of whey protein by nanofiltration. International Dairy Journal. 2013; 28(2):94-101.
[97] Song R, Wei RB, Luo HY, Wang DF. Isolation and characterization of an antibacterial peptide fraction from the pepsin hydrolysate of half-fin anchovy (*Setipinna taty*). Molecules. 2012; 17(3):2980-2991.
[98] Balakrishnan B, Prasad B, Rai AK, Velappan SP, Subbanna MN, Narayan B. In vitro antioxidant and antibacterial properties of hydrolysed proteins of delimed tannery fleshings: comparison of acid hydrolysis and fermentation methods. Biodegradation. 2011; 22(2):287-295.
[99] Torruco-Uco J, Chel-Guerrero L, Martínez-Ayala A, Dávila-Ortíz G, Betancur-Ancona D. Angiotensin-I converting enzyme inhibitory and antioxidant activities of protein hydrolysates from *Phaseolus lunatus* and *Phaseolus vulgaris* seeds. LWT-Food Science and Technology. 2009; 42(10):1597-1604.
[100] Minervini F, Algaron F, Rizzello CG, Fox PF, Monnet V, Gobbetti M. Angiotensin I-converting-enzyme-inhibitory and antibacterial peptides from Lactobacillus helveticus PR4 proteinase-hydrolyzed caseins of milk from six species. Applied and Environmental Microbiology. 2003; 69(9):5297-5305.
[101] Karnjanapratum S, O'Callaghan YC, Benjakul S, O'Brien N. Antioxidant, immunomodulatory and antiproliferative effects of gelatin hydrolysate from unicorn leatherjacket skin. Journal of the Science of Food and Agriculture. 2016; Journal of the Science of Food and Agriculture, 96, 3220–3226.

Plant extracts as a natural corrosion inhibitors of metals and its alloys used in food preserving industry

A. Ninčević Grassino

Department of Chemistry and Biochemistry, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia

The protection of metals and its alloys against corrosion has attracted great attention, due to their various industrial application and economic importance. Pure metals and its alloys react chemically or electrochemically with corrosive medium to form stabile corrosion compounds in which the loss of metals occurs and consequently metal surface becomes corroded. In order to prevent corrosion damage and eventual failure of components and systems both in processing and manufacturing industries, the use of corrosion inhibitors is very popular. Corrosion inhibitors are synthetic or natural substances when added in small concentrations to corrosive media inhibited the corrosion reaction and reduced corrosive attack on metals. Although, many synthetic compounds showed good anticorrosive properties, most of them are toxic for human and environmental. Due to the fact that increasing the awareness of health and ecological risks, new eco-friendly and harmless natural anticorrosive substances have drawn great attention. The various plants, such as aromatic herbs and spices have been used not only by human beings, e.g. food-stuffs, flavour, fragrance and medicine, but also as a rich source of anticorrosive inhibitors. Plants inhibition performance and efficiency are related to their chemical composition, which take the form of alkaloids, carbohydrates, polyphenols, proteins and tannins. These compounds usually have functional groups with oxygen, nitrogen and sulphur atoms, which can be easy adsorbed on metal surface due to range of functional group properties.
Therefore, this work reports application of plant extracts as anticorrosive agents for metals and its alloys used in food preserving industry. Furthermore, the methods used for extraction and characterization of plant materials, as well as methods for calculation of corrosion inhibition efficiency are described, too.
In conclusion, the propose work points out that plants extract can be considered as a promising industrial resource for protection of metals and its alloys due to theirs environmental acceptability, ready availability and high inhibition efficiency properties.

Keywords: corrosion; natural inhibitors; plant extracts; chromatographic, spectroscopic and electrochemical techniques; surface analyses

1. Introduction

Steel, aluminium, tin and chromium are four metals commonly used for the food packaging. Tin and steel, chromium and steel are used as composite materials in the form of tinplate and electrolytic chromium-coated steel (ECCS), the letter being referred as a tin free steel (TFS). Aluminium is used in the form of purified alloys containing small and carefully controlled amounts of magnesium and manganese. These metals are important materials for food packaging, due to several important qualities, i.e. good mechanical strength, toughness, ductility, solderability, weldability, nontoxicity, lubricity, lacquerability and impermeability. However, the chemical structure that gives them practical properties is also responsible for their main weakness, i.e. their susceptibility to corrosion. Corrosion is term used to describe chemical/electrochemical reaction between metal and its environment to form corrosive compounds to some extent. When the metallic surface is not compact and continuous, the metal is exposed through the pores to aggressive food compounds or food additives and its corrosion is accelerated. The factors which may influence the corrosion are choice or damage of metallic surface, passivation level, type of food product, pH and acid content, presence of corrosion accelerators (nitrate, sulphur and phosphate compounds, plant pigments and synthetic colouring in the food), presence of oxygen within the sealed can, thermal processing (heat sterilization treatment) and storage temperature and time. The consequences are dissolution of high levels of metals, i.e. tin, iron, chromium and aluminium into food content [1-3]. For example, when tin and iron are in excess, they are toxic and may cause gastrointestinal symptoms provoking nausea, vomiting, diarrhea, fever and headache. The maximum limit for tin in canned solid food is 250 mg/kg and 150 mg/kg for beverages, while for iron the values are 50 mg/kg [4].

Due to the fact that corrosion reaction takes place at the metal surface, the rate of attack (corrosion rate) can be reduced and controlled by modifying the conditions at the surface. In this context, different corrosion inhibitors are recommended, nowadays particularly a new group of natural products is of interest, due to low toxicity, eco-friendliness and good corrosion efficiency. A number of papers reported in recent years the use of different natural products for corrosion inhibition of steel and aluminium, such as rosemary, lavender, ginkgo, laurel, thyme, zingiber, mentha and ruta [5-14]. However, a limited numbers of research showed application of natural products as corrosion inhibitors of tinplate and tin free steel, commonly used material for food container manufacture. Ninčević Grassino *et al*. were reported the use of essential onion oil (EOO) as a potential inhibitor of tin, chromium and iron from tinplate sheets [15,

16]. Head-space gases (H_2, O_2, N_2 and CO_2), as indicators of corrosion process were analysed by gas chromatography [16], whereas dissolution of iron and tin from tinplate can filled with tomato purée in presence of EOO and potassium nitrate were studied using atomic absorption spectroscopy and high performance liquid chromatography [17]. Furthermore, chemical composition. i.e. sugar, organic and amino acids content in canned tomato purée with EOO were analysed by high performance liquid chromatography [18].

Several studies have been shown that peels and seeds from various plant, as well as by-products or waste derived from food industry can be used for isolation and application of different value added compounds as a potential corrosion inhibitor. The extracts from orange, mango, passion fruit, cashew and tomato peel [19-21], *Garcinia* Kola and *Pongamia pinnata* seeds [22, 23] and grape pomace [24] have already shown promising and efficient corrosion inhibition properties.

In conclusion, the recent trend of reporting plant extracts as corrosion inhibitor of mild and carbon steel can be carried out on the tinplate, tin free steel and aluminium, due to the fact that these materials are not investigated enough. In addition, novel, economical and eco-friendly corrosion inhibitor derived from cheap and abundant renewable resources offer other enormous opportunities for further investigations.

2. Materials used for can production

Tinplate used for food packaging is formed of steel base, tin-iron alloy, free tin and passivated film with tin oxides, metallic chromium and chromium oxide. The steel base is a low carbon mild steel with 0.03-0.13 % of carbon. In general, its chemical composition has an important role in corrosion resistance of tinplate. The carbon steel base is coated with pure tin on both faces of sheets by hot dipping or anodic electrolytic processes in a molten tin.

In general, electroplating can be defined as an electrolytic metal deposition due to reduction reaction on the cathode electrode where the thin layer of metal adhered as a surface finishing processing. Thin metal films are plated on metals and alloys surface to enhance their appearance, corrosion and wear resistance. Such films must be adherent and uniform on regular or irregular metal surface, it must be cleaned prior to electro deposition in order to remove various foreign substances, e.g. oils, grease, dirt, oxides, etc. Otherwise, poor film adhesion and incomplete deposition will not protect the base steel and appearance will not be so satisfactory.

The main advantage of tin electrolytic deposition is the possibility of providing a higher coating weight, which will be in contact with aggressive food products [25]. Normally, this coating weight is used for the manufacture of internal surface of containers. It should be noted that application of heavier coating weight on internal and lighter coating weight on external surface of tinplate is the common practice in the food canning industry. This kind of material is called differentially coated tinplate with tin grades from 2.8 to 11.2 g/m^2. The tin-iron alloy ($FeSn_2$) is produced by diffusion of pure tin into the steel base during electrolytic process [26]. When the alloy layer is not compact and continuous, the steel is exposed through the pores to aggressive food compounds and its corrosion is accelerated.

During production of the tinplate, tin oxide films may be formed on its surface [27]. To prevent uncontrolled oxidation of the tin and protect metal against further oxidation, the tinplate is subjected to passivation treatment [28]. Also, it is applied to prevent appearance of sulphide stains by certain canned food (meat, fish and some vegetable products) and to improve lacquer adhesion to the metal substrate, i.e. the corrosion resistance towards food media. The most widely used passivation treatments are cathodic or a simple chemical dip treatment in a solution of sodium dichromate. Due to the fact that chromium compounds used in these procedures are toxic and carcinogenic, therefore diverse alternative passivation treatments were studied [29-31]. However, the conventional chromium passivation treatment is still used.

Besides tinplate, tin free steel (TFS) is also produced by electroplating, i.e. applying electrolytic chromic acid treatment over steel sheets. This type of material was developed to meet economic requirement and overcomes some tinplate properties. TFS shows great paint adhesion, excellent resistance to black sulphide stain and corrosion resistance after painting. Furthermore, no discoloration or deterioration causes by high temperature are the main properties of TFS.

It should be mentioned that some of non electrolytic finishing process include aluminium anodizing, i.e. chemical reaction between aluminium and oxygen to form a thin aluminium oxide film on an aluminium base metal. This process occurs as a natural phenomenon in air. Aluminium tendency to form passivation oxide layer, results in simple and ease manufacture of single piece aluminium cans, compared to laboriously constructed three pieces of steel. Besides single-serving containers, aluminium is the favoured material for shaped food cans, e.g. rectangular, oval, square, etc. Formable and versatile, aluminium offers opportunities for enhanced aesthetic appeal. Aluminium is less costly than tinplated steel, but offers the same resistance to corrosion. In addition, aluminium shows low density, good electrical and thermal conductivities and high ductility and good corrosion resistance.

3. Corrosion

Corrosion is defined as s destructive attack on metals and alloys through chemical or electrochemical reaction of metallic surface with corrosive medium. The consequence of its interactions is movement of metal ions into the solution at active areas (anode), passage of electrons from the metal to an acceptor at less active areas (cathode), an ionic current in the solution and an electronic current in the metal. In other words, corrosion involves formation of stabile compounds, i.e. corrosion products, between the compounds of corrosive solution and metals or its alloys. The tendency of a metal to corrode depends on different factors, such as structure of metal and its composition formed during alloying, damage of metal surface developed during fabrication, type of corrosion medium, pH and acid content, presence of corrosion accelerators (oxygen, nitrate, sulphur and phosphate compounds in corrosion medium), temperature and time of metal/metal alloys contacts with aggressive corrosion medium. According to work obtained by Chigondo and Chigondo [32], the different types of corrosion can be recognised, i.e. uniform, pitting, stress corrosion fatigue, intergranular crevice, filiform, erosion and fretting. The classification was done depending on environmental surrounding of metal, type of metal or metal alloys and possible chemical reactions occurred.

4. Corrosion prevention

Due to the fact that corrosion presents ubiquitous problem for a wide range of industrial applications and products, different strategies were employed to avoid possible metals dissolution and consequently contamination of foodstuffs. Corrosion progress can be prevented by lacquer coating application onto metallic surface or by addition of small amounts of corrosion inhibitor to corrosive medium.

4.1 Lacquer corrosion prevention

The use of organic coatings, i.e. lacquers on tinplate, tin free steel and aluminium has become a generalised procedure for cans manufacture. Lacquers should be chemically inert, resistant to mechanical or thermal stress and must adhere completely on metallic surface. Usually, one or two layers of lacquers are applied on metal surface to prevent interaction between the cans and its contents [33-35]. In some cases loss of adhesion may occur due to damage on the can surface during fabrication, but more often the detachment of the coating is due to breakdown processes taking place through or under the coating, due to the corrosion spreading from exposed metal. Therefore, the monitoring of lacquers [36, 37] is of major interest in industry practices in order to reduce the risk of loss of product properties and /or product contamination.

4.2 Synthetic organic corrosion inhibitors

Instead lacquers, there is a great interest of using different synthetic heterocyclic organic compounds, which are acting as a cathodic or anodic corrosion inhibitors. It has been found [38-41] that synthetic organic compounds could be used as effective corrosion inhibitors, due to the fact that they contained heteroatoms with high electron density and double or triple bonds in structure. Obviously, the compounds with both nitrogen and sulphur in their molecular structure have shown excellent corrosion ability, compared to those containing only nitrogen or sulphur. Moreover, the sulphur containing compounds possessed stronger corrosion inhibition efficiency, compared to nitrogen containing compounds. Inhibition function of organic compounds depends on its physicochemical properties, such as presence of functional groups, possible steric effects, electronic density of donor atoms and the possible interaction of p-orbitals of the inhibitor with d-orbitals of the metal surface atoms.

Generally, corrosion inhibitions protection is achieved by adsorption of ions or molecules of inhibitors onto metal surface. The absorption mechanism involves four possible types: *i*) electrostatic attraction between charged molecules of inhibitors and charged metal surface, *ii*) interaction of unshared electron pairs of the molecules with the metal surface, *iii*) interaction of the presence of conjugated bonds (π electron) in the compound with metal, and *iv*) adsorption occur by combination of *i*) and *iv*). As a results of adsorption, the corrosion rate is reduce by increasing or decreasing the anodic and/or cathodic reaction, decreasing the diffusion rate of aggressive components to the metal surface and decreasing the electrical resistance of the metal surface [42]

Adsorption mechanism of organic inhibitor at metal/solution interface, firstly involves replacement of one or more water molecules, initially adsorbed at the metal surface (Eq. 1).

$$Inh_{(sol)} + xH_2O_{(ads)} \rightarrow Inh_{(ads)} + xH_2O_{(soln)} \quad \text{Eq. 1}$$

$Inh_{(soln)}$ and $Inh_{(ads)}$ represent the inhibitors in the solution and adsorbed on the metal surface, respectively and x is the number of water molecules displaced by inhibitor.

Subsequently, the inhibitor may reacts with metal ions (M^{2+}), generated by oxidation or dissolution process (Eq. 2). Depending on relative solubility, the forming metal inhibitor complex (Eq. 3) may inhibit or catalyse further metal dissolution.

$$M \rightarrow M^{2+} + 2e^- \quad \text{Eq. 2}$$

$$M^{2+} + Inh_{(ads)} \rightarrow [M\text{-}Inh]^{2+}_{(ads)} \quad \text{Eq. 3}$$

Although, synthetic organic compounds are efficient corrosion inhibitors, their synthesis is not always simple and cost-effective and many of them are toxic for humans and environment.

4.3 Natural organic corrosion inhibitors

Based on environmental and safety requests, the new class of non-toxic, natural corrosion inhibitors were developed. Natural products have potential to replace synthetic organic inhibitors due to follow main advantages, i.e. environmentally friendly and biodegradable in nature, readily available, synthesis by simple procedure with low cost. They are used for flavouring some food products, too, but their application in food canning industry as a corrosion inhibitor is not investigated enough. A number of natural products of plant origin, such as fruit, leaves, peel and seeds extracts have been reported as anticorrosive agents of various metals and alloy in acidic media, but their application as corrosion inhibitor for tinplate, tin free steel and aluminium used for production of food containers have not been studied yet. In addition, by-products or waste derived from food processing industry could be also used as a promising industrial resource for bio-compounds production and application as a non-toxic, cheap and effective green corrosion inhibitor, instead of ordinary chemical and toxic inhibitors.

As was previously mentioned, adsorption is main mechanism for most of the synthetic inhibitors. The inhibitive properties of natural, eco-friendly inhibitors are also based on two types of adsorption processes, i.e. physical (physisorption) and chemical (chemisorption) adsorptions. Physical adsorption occurred due to the electrostatic, dipole-dipole interactions between charged molecules of inhibitor and charged metal surface. Chemical adsorption involves the transfer or shearing of electrons from inhibitor to the metallic surface, resulting in formation of co-ordinate bond.

For example, the work obtained by Liu *et al*. [43] have shown that bamboo leaf extracts manifest both, physisorption and chemisorption mechanisms, at extract concentrations of 10 - 80 mg/L and 90 - 200 mg/L, respectively in hydrochloric and sulphuric acids. They have shown that bamboo leaf extract can be used as excellent natural inhibitor of cold rolled steel due to presence of O and N atoms in functional groups (O-H, N-H, C-C, C-O, C-N, C-N, C-O) and aromatic ring. Furthermore, the results of FTIR spectroscopy have also confirmed that *Ginko* leaves extracts contained oxygen and nitrogen atoms in mentioned functional groups [9]. Therefore, protection of metallic surface is done via O and N atoms presented in the flavonoids, ginkgolides and amino acids as main constituents of bamboo and *Ginko* leave extracts. Oxygen and nitrogen atoms found in the functional groups, such as C=O, N-H, O-H, C-O, C-N, C=C and aromatic ring of *Ilex kudingcha* C.J. Tseng are also responsible for the inhibition of the corrosion reaction on J55 steel [44]. Furthermore, FTIR and dispersive X-ray spectroscopy (EDX) results showed that *Piper longum* fruit extract contains oxygen and nitrogen atoms in functional groups (O-H, C=C, C=O, C-N, C-O) and aromatic ring, which meets the general consideration of typical corrosion inhibitors [23]. In addition, phenolic compounds, particularly flavonoids [20, 24, 45] have been shown to possess significant corrosion inhibition efficiency, which is primarily in relationship with their structural characteristics, such as number and position of phenolic hydroxyle or other groups and conjugation. Flavonoids are especially important antioxidants due to their high redox potential, which allows them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They possessed metal chelating potential, forming bidentate metal chelates at the ortho-diphenolic groups of rings B and C [46, 47].

Phytochemical screening carried out on the extracts from *Phyllanthus fraternus* [48] and *Saccocalyx satureioides* [49] are constituent alkaloids, saponins, tannins, terpenoids, steroids, flavonoids and phenols. The corresponding chemical structures of these compound reveals that they contain O in functional groups, aromatic rings and O-heterocyclic rings, which encounter the general characteristics of typical corrosion inhibitors. Furthermore, analysis of *Ruta chalepensis* oil [14], using gas chromatography and gas chromatography/mass spectroscopy revealed that the major components responsible for corrosion inhibition were 2-Undecanone (67 %), 2-Decanone (9 %), 6-(3',5'-Benzodioxyl)-2-hexanone (6.3 %) and 2-Dodecanone (4 %).

5. Methods used for extraction of plant materials

Extraction of plant material can be done using various methods, divided into two main groups, i.e. conventional or un-conventional (Fig. 1). All these techniques have shown some common objectives: *i*) to extract target compounds from complex plant material, *ii*) to convert the target compounds into a more suitable form for separation, detection and

application and *iii*) to provide strong and reproducible separation method that is independent of variations in the sample matrix. The extracting power of conventional (classical) techniques is based on application of heat and/or mixing of plant material with different solvents in use. Compare to conventional, non-conventional methods are more environmental friendly due to decreased use of organic chemical, reduced time of extraction, provided better yield and quality of extracts. It is important to mention that understanding of every aspect of used non-conventional techniques is crucial for successful isolation of target compound, due to the fact that most of these methods are based on different mechanism. However, to compare success of newly developed methods, the conventional ones are still considered as the reference method.

The majority of plant extracts for corrosion inhibition purposes were prepared by refluxing method [23, 43, 48, 50 - 52], such as *Piper longum* (distilled water, 5 h), bamboo (80 %, *v*/*v* ethanol, 75 °C, 2 h), *Phyllanthus fraternus* (1 M H_2SO_4, 5 h), *Gundelia tournefortii* (7:3 *v*/*v*, methanol/water mixture, 80 ºC, 1 h), eggplant peel (distilled water, 2 h) and *Salvadora persica* (distilled water, 5 h). Furthermore, the peels of mango and orange [20] and seeds of *Garcinia kola* [22] were extracted in a Soxhlet extractor using ethanol, ethyl acetate, hexane and absolute ethanol, respectively. Other extracts, e.g. *Laurus nobilis*, *Ilex paraguariensis*, *Saccocalyx satureioides*, *Cola Acuminata*, *Camellia Sinensis*, *Ocimum gratissimum*, *Menta pulegium*, *Lavandula dentate*, *Matricaria recutita* and grape pomace were prepared by boiling, soaking, continuous agitation, hydro-distillation, ultrasonication and steam distillation [8, 10, 13, 26, 49, 53 - 55].

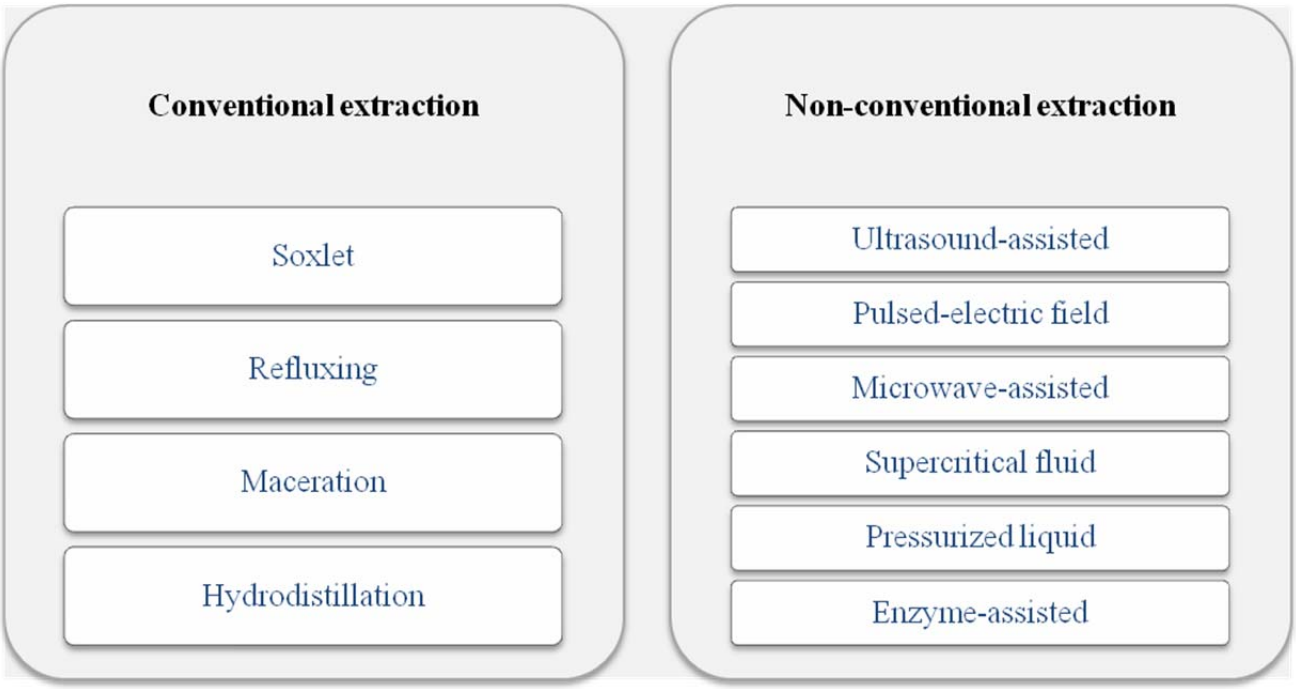


Fig. 1 Extraction method used for preparation of plant extracts.

6. Methods used for evaluation of corrosion inhibition efficiency

In order to investigate the inhibition efficiency of plant extracts in various corrosive medium, several methods have been employed. Frequently used are weight loss or gravimetric analysis and electrochemical methods. Weight loss test is base on sheets or specimens weight measurements before and after theirs immersion in corrosive medium during certain period. Subsequently, corrosion rate and efficiency can be calculated using specimen weight loss (Δm), specimen surface (A) and immersion time (t).

Electrochemical kinetics of a metal exposed to a corrosive medium (electrolyte) can be characterized by determining at least three polarization parameters, such as corrosion current density, (i_{corr}) corrosion potential (E_{corr}) and Tafel slopes (β_a and/or β_c). The rate of metal dissolution or corrosion can be calculated using follow polarization methods:

- Linear polarization which covers both anodic and cathodic portions of the potential (E) versus current density (i) curve for determination of polarization resistance (R_p).
- Tafel extrapolation technique which takes into account linear parts of the anodic and cathodic curves for determination of R_p. This method involves determination of the Tafel slopes β_a and/or β_c, as well as E_{corr} and i_{corr} from a single polarization curve.
- Electrochemical impedance spectroscopy requires alternating current and the output is Nyquist plot for charge transfer or diffusion control process, which can be used to determine R_p, which in turn is inversely proportional to the corrosion current density i_{corr}.

All mentioned electrochemical methods were used Li *et al*. [43] for determination of bamboo leaf extracts inhibition efficiency on cold rolled steel in two corrosion media, i.e. sulphuric (0.5 - 5.0 M) and hydrochloric (1.0 - 5.0 M) acids. They were found that inhibition efficiency increases due to increases of extracts concentration (5 - 200 mg/L) and immersion time (6 - 32 h). Maximum inhibition efficiency of 89 % and 79 % was obtained in 1.0 M hydrochloric and 0.5 M sulphuric acids, respectively. Deng and Li [9] were also investigated the inhibition effect of *Ginko* leaves extracts in hydrochloric (1.0 - 5.0 M) and sulfuric (0.5 - 2.5 M) acids using weight loss, potentiodynamic polarization and electrochemical impedance spectroscopy measurements. The results showed that *Ginko* leaves extract act as a good corrosion inhibitor, particularly in 1.0 M hydrochloric compared to 0.5 M sulphuric acid. In addition, they were used atomic force microscope (AFM) and scanning electron microscopy (SEM) analyses as a two powerful methods to investigate the surface morphology at nano to microscale. These techniques have become a new choice to study the influence of inhibitors on the generation and progress of corrosion at the metal-solution interface. AFM and SEM techniques were also used Nasibi *et al*. [46] for mild steel surface examination after interaction with chamomile extracts. For determination of inhibition efficiency, they were applied electrochemical, i.e. potentiodynamic polarization and impedance spectroscopy techniques, which have shown that chamomile extracts acts as excellent inhibitor (93.3 %), at temperature of 22 °C and concentration of 7.2 g/L. Furthermore, corrosion inhibition efficiency of green tea extracts tested by weight loss, potentiodynamic polarization and electrochemical impedance spectroscopy has shown that all employed techniques are in good agreement with each other [45]. Maximum inhibition efficiency was 81.5 % (1 M HCl) and 71.7 % (0.5 M H_2SO_4), at extract concentration of 500 ppm. The inhibition efficiency of *Gundelia tournefortii* extract was found to be 93 % and 90 % in 2.0 M HCl and 1.0 M H_2SO_4, respectively, at concentration of 150 ppm [50]. Cathodic and anodic polarization curves show that *G. tournefortii* extract is a mixed-type inhibitor in both acidic media. In this context, the electrochemical Tafel polarization studies [56] revealed that *Eriobotrya japonica* (EB) leaf extract also acts as mixed type of inhibitor in hydrochloric (0.5 M), whereas in sulphuric (0.5 M) acid as anodic type. The impedance response of EB extracts consisted of characteristic depressed semicircles clarifying that the corrosion process of mild steel occurs under charge transfer control, while thermodynamic parameters indicated that the inhibition of corrosion occurred by physical adsorption mechanism. Furthermore, the work done by Bouammali *et al*. [8] showed that aqueous extract of *Lavandula dentata* in 1 M HCl also acts as mixed type with maximum inhibition efficiency of 95 % obtained at mass fraction of 2 %. Other aromatic herbs such as *Mentha pulegium* and *Ruta chalepensis* [13, 14] were proved as a efficient corrosion inhibitors on mild steel in acid media. The inhibitory effect of *Mentha pulegium* extract was found to increase with the concentration and reached 88 % at 33 % (*v*/*v*), whereas for *Ruta chalepensis* oil attained 77 % at 2.5 ml/L. In addition, *Thymus vulgaris*, *Xylopia aethiopica*, *Zingiber officinale*, *Piper longum*, *Saccocalyx satureioides*, *Cola acuminata* and *Camellia sinensis* leaves mix extracts, *Ocimum gratissimum*, *Larrea Tridentata*, *Eulychnia acida* Phil., *Khaya senegalensis*, punica plant and *Thapsia villosa* have been also successfully applied as a eco-friendly and non-toxic corrosion inhibitor of steel in acid media confirmed by already mentioned methods [23, 49, 54, 55, 57 - 61].

In the conspectus of using natural products as a corrosion inhibitor, various plant peels, seeds and pomace have been investigated as a cheap and effective corrosion inhibitor of mild and carbon steel. In this context, the extracts from mango, orange, passion fruit and cashew peels [19, 20], eggplant peel [51], *Cucurbita maxima* peel [62], *Musa acuminata* [63], have been tested, using electrochemical methods, weight loss measurements and SEM surface analysis. They have shown inhibition efficiencies in the range of 48 to 96 %, depending on peel type, extract concentrations, time and temperature of immersion and method used for corrosion assay. Furthermore, the grape pomace, as an industrial waste from wine and juice processing industry was also evaluated as corrosion inhibitor of steel. The maximum inhibition efficiency of 83 % and 93 % was obtained for 3%, crude and concentrated extracts.

7. Conclusion

Various plants extract are found to be effective, eco-friendly and non-toxic corrosion inhibitor due to presence of different organic compound, such as alkaloids, flavonoids, phenols, saponins, steroids, tannins, terpenoids, etc, whose attachment to adsorption sites on the metal surface is responsible for the inhibition process. Theirs excellent protection of steel in acidic medium was confirmed in different scientific work. However, its inhibition performance were not proved enough on tinplate, tin free steel and aluminium, commonly used materials in food canning industry. Moreover, theirs inhibition action were not investigated in other corrosive medium, except acidic, mostly hydrochloric and sulphuric. Therefore, it is certain that in the coming years usage of plant extracts can be promising, not yet fully exploring material for future scientific investigation. Furthermore, by-products or waste derived from food processing industry can be also propose as a new alternative natural resource for production of compounds with anticorrosive properties.

References

[1] Perring, L, Basic-Dvorzak, M. Determination of total tin in canned food using inductively coupled plasma atomic emission spectroscopy. *Analytical and Bioanalytical Chemistry*. 2002;374:235-243.

[2] Boogaard, PJ, Boisset, M, Blunden, S, Davies, S, Ong, TJ, Taverne, JP. Comparative assessment of gastrointestinal irritant potency in man of tin (II) chloride and tin migrated from packaging. *Food and Chemical Toxicology*. 2003;41:1663-1670.

[3] Shimbo, S, Matsuda-Inoguchi, N, Watanabe, T, Sakurai, K, Date, C, Nishimura, A, Nakatsuka, H, Saito, H, Arisawa, K, Ikeda, M. Dietary intake of tin in Japan, and the effects on intake of canned food and beverage consumption. *Food Additives and Contaminants*. 2007;24:535-545.

[4] European Commission. 2002. Commission Regulation (EC) No. 562/2002 of 2nd April 2002: setting maximum levels for certain contaminants in foodstuffs. *Official Journal of European Community*. L 86/5.

[5] Kliškić, M, Radošević, J, Gudić, S, Katalinić, V. Aqueous extract of *Rosmarinus officinalis* L. as inhibitor of Al-Mg alloy corrosion in chloride solution. *Journal of Applied Electrochemistry*. 2000;30:823-830.

[6] Vrsalović, L, Kliškić, M, Gudić, S. Application of Phenolic Acids in the Corrosion Protection of Al-0.8Mg Alloy in Chloride Solution. *International Journal of Electrochemical Science*. 2009;4:1568-1582.

[7] Halambek, J, Berković, K, Vorkapić-Furač, J. The influence of *Lavandula angustifolia* L. oil on corrosion of Al-3Mg alloy. *Corrosion Science*. 2010;52:3978-3983.

[8] Bouammali, H, Ousslim, A, Bekkouch, K, Bouammali, B, Aouniti, A, Al-Deyab, SS, Jama, C, Bentiss, F, Hammouti, B. The Anti-Corrosion Behavior of *Lavandula dentata* Aqueous Extract on Mild Steel in 1M HCl. *International Journal of Electrochemical Science*. 2013;8:6005-6013.

[9] Deng, S, Li, X. Inhibition by *Ginkgo* leaves extract of the corrosion of steel in HCl and H_2SO_4 solutions. *Corrosion Science*. 2012;55:407-415.

[10] Gülşen, A. Corrosion inhibition of mild steel by *Laurus nobili*s leaves extract as green inhibitor. *Research on Chemical Intermediates*. 2012;38:1311-1321.

[11] Halambek, J, Cvjetko Bubalo, M, Radojčić Redovniković, I, Berković, K. Corrosion Behaviour of Aluminium and AA5754 Alloy in 1% Acetic Acid Solution in Presence of Laurel Oil. *International Journal of Electrochemical Science*. 2014;9:5496-5506.

[12] Okafor PC, Apebende EA. Corrosion inhibition characteristics of *Thymus vulgaris*, *Xylopia aethiopica* and *Zingiber officinale* extracts on mild steel in H_2SO_4 solutions. *Pigment & Resin Technology*. 2014;43:357-364.

[13] Khadraoui, A, Khelifa, A, Boutoumi, H, Belkheir, H. *Mentha pulegium* extract as a natural product for the inhibition of corrosion. Part I: electrochemical studies. *Natural Product Research*. 2014;28:1206-1209.

[14] Khadraoui, A, Khelifa, A, Boutoumi, H, Hamitouche, H, Mehdaoui, R, Al-Deyab, SS. Adsorption and Inhibitive Properties of *Ruta chalepensis* L. Oil as a Green Inhibitor of Steel in 1 Hydrochloric Acid Medium. *International Journal of Electrochemical Sc*ience. 2014;9:3334-3348.

[15] Ninčević Grassino, A, Grabarić, Z, Pezzani, A, Fasanaro, G. Lo Voi, A. Influence of essential onion oil on tin and chromium dissolution from tinplate. *Food and Chemical Toxicology*. 2009;47:1556-1561.

[16] Ninčević Grassino, A, Grabarić, Z, Pezzani, A, Squitieri, G, Berković, K. Corrosion inhibition with different protective layers in tinplate cans for food preserving. *Journal of the Science of Food and Agriculture*. 2010;90:2419-2426.

[17] Ninčević Grassino, A, Grabarić, Z, Pezzani, A, Squitieri, G. Fasanaro, G, Impembo, M. Corrosion behaviour of tinplate cans in contact with tomato purée and protective (inhibiting) substances, *Food Additives and Contaminants*. 2009;26:1488-1494.

[18] Ninčević Grassino, A, Grabarić, Z, De Sio, F, Cacace, D, Pezzani, A, Squitieri, G. Effect of storage time and natural corrosion inhibitor on carbohydrate and carboxylic acids content in canned tomato purée. *Food Science and Technology International.* 2012;18**:**219-22.

[19] da Rocha, JC, da Cunha Ponciano Gomes, JA, D'Elia, E. Corrosion inhibition of carbon steel in hydrochloric acid solution by fruit peel aqueous extracts. *Corrosion Science*. 2010;52: 2341-2348.

[20] da Rocha, JC, da Cunha Ponciano Gomes, JA, D'Elia, E. Aqueous Extracts of Mango and Orange Peel as Green Inhibitors for Carbon Steel in Hydrochloric Acid Solution. *Materials Research*. 2014; 17:1581-1587.

[21] Ninčević Grassino, A, Halambek, J, Rimac-Brnčić, S, Djaković, S, Dent, Grabarić, Z. Utilization of tomato peel waste from canning factory as a potential source for pectin production and application as tin corrosion inhibitor. *Food Hydrocolloids*. 2016;52:265-274.

[22] Ikeuba, AI, Okafor, PC, Ekpe, UJ, Ebenso, EE. Alkaloid and Non-Alkaloid Ethanolic Extracts from Seeds of *Garcinia Kola* as Green Corrosion Inhibitors of Mild Steel in H_2SO_4 Solution. *International Journal of Electrochemical Science*. 2013;8:7455-7467.

[23] Singh, A, Singh, VK,·Quraishi, MA. Inhibition of Mild Steel Corrosion in HCl Solution Using Pipali (*Piper longum*) Fruit Extract. *Arabian Journal of Science and Engineering*. 2013;38:85-97.

[24] da Rocha, JC, Ponciano Gomes, JAC, D'Elia, E, Gil Cruz, AP, Cabral, LMC, Torres, AG, Monteiro, MVC. Grape Pomace Extracts as Green Corrosion Inhibitors for Carbon Steel in Hydrochloric Acid Solutions. *International Journal of Electrochemical Science*. 2012;7:11941-11956.

[25] Zhu, X, Sandenberg, RF. Corrosion of tinplate T54S and T61 in humid atmosphere and saline solution. *Material Corrosio*n. 2001;52:685-690.

[26] Elias, RA, Forder, SD, English, TH, Breen, C. ^{57}Fe conversion electron Mössbauer spectroscopy of factors influencing Fe-Sn intermetallic phase formation in tinplated steel. *Hyperfine Interaction*. 2002;141/142:447-451.

[27] Zurlini, C, Gelati, S, Fragni, R, Montanari, A. Determination of tin oxides on the surface of electrolytic tinplate. *Industria Conserve*. 2003a;78: 313-318.

[28] Zurlini, C, Fragni, R, Ferretti, C, Montanari, A, Chiodi, D. 2003b. Comparison of methods for chromium determinations in tinplate passivation film. *Industia Conserve*. 2003b;78:293-309.

[29] Arenas, MA, Conde, A, de Damborenea, JJ. Cerium: a suitable green corrosion inhibitor for tinplate. *Corrosion Science*. 2002;44:511-520.
[30] Mora, N, Cano, E, Polo, JL, Puente, JM. Corrosion protection properties of cerium layers formed on tinplate. *Corrosion Science*. 2004;46:563-578.
[31] Catalá, R, Alonso, M, Gavara, R, Almeida, E, Bastidas, JM, Puente, JM, de Cristaforo, N. Titanium-passivated tinplated for canning food. *Food Science and Technology International*. 2005;11**:**223-227.
[32] Chigondo, M, Chigondo, F. Recent Natural Corrosion Inhibitors for Mild Steel: An Overview. *Journal of Chemisty*. 2016, Article ID 6208937:7 pages. http://dx.doi.org/10.1155/2016/6208937.
[33] Barilli, F, Fragni, R, Gelati, S, Montanari A. Study on the adhesion of different types of lacquers used in food packaging. *Progress in Organic Coatings*. 2003;46:91-96.
[34] Bernardo, PEM, dos Santos, JLC, Costa, NG, Influence of the lacquer and end lining compound on the shelf life of the steel beverage can. *Progress in Organic Coatings*. 2005; 54**:**34-42.
[35] Manfredi, LB, Ginés, MJL, Benítez, GJ, Egli, WA, Rissone, H, Vázquez, A. Use of epoxy phenolic lacquers in food can coatings: characterization of lacquers and cured films. *Journal of Applied Polymer Science*. 2005;95:1448-1458.
[36] Ninčević, A, Pezzani, A, Squitieri, G. Characterization of different types of lacquers used in food packaging: porosity, flexibility and IR reflectance spectroscopy tests. *Acta Alimentaria*. 2006;35:397-407.
[37] Ninčević, A, Pezzani, A, Squitieri, G. Characterization of different types of lacquers used in food packaging: lacquer adhesion tests. *Acta Alimentaria*. 2007;36:27-37.
[38] Bentiss, F, Traisne, M, Lagrenée, M. The substituted 1,3,4-oxadiazoles: a new class of corrosion inhibitors of mild steel in acidic media. *Corrosion Science*. 2000;42:127-146.
[39] Morad, MSS, Hermas, AAA, Influence of some amino acids and vitamin C on the anodic dissolution of tin in sodium chloride solution. *Journal of Chemical Technology and Biotechnology*. 2001;76**:**401-410.
[40] Migahed, MA, Azzam, EMS, Al-Sabagh, AM, Corrosion inhibition of mild steel in 1 M sulfuric acid solution using anionic surfactant. *Matererials Chemisty and Physics*. 2004;85:273-279.
[41] Mu, G, Li, X, Inhibition of cold rolled steel corrosion by Tween-20 in sulphuric acid: weight loss, electrochemical and AFM approaches. *Journal of Colloid and Interface Science.* 2005:289:184-192.
[42] Khan, G, Newaz, KMS, Basirun, WJ, Ali, HBM, Faraj, FL, Khan, GM. Application of Natural Product Extracts as Green Corrosion Inhibitors for Metals and Alloys in Acid Pickling Processes - A review. *International Journal of Electrochemical Science*. 2015;10:6120-6134.
[43] Li, X, Deng, S, Fu, H. Inhibition of the corrosion of steel in HCl, H_2SO_4 solutions by bamboo leaf extract. *Corrosion Science*. 2012;62:163-175.
[44] Chen, S, Singh, A, Wang, Y, Liu, W, Deng, K, Lin, Y. Inhibition effect of Ilex kudingcha C.J. Tseng (Kudingcha) extract on J55 Steel in 3.5wt% NaCl Solution Saturated with CO_2. *International Journal of Electrochemical Science*. 2017;12:782-796.
[45] Alsabagh, AM, Migahed, MA, Abdelraouf, M, Khamis, EA. Utilization of Green Tea a Environmentally Friendly Corrosion Inhibitor for Carbon Steel in acidic media. *International Journal of Electrochemical Science*. 2015;10:1855-1872.
[46] Nasibi, M, Zaarei, D, Rashed, G, Ghasemi, E. Chamomile (Matricaria recutita) Extracts as a Corrosion Ihibitor for Mild Steel in Hydrochloric Acid Solution. *Chemical Engineering Communications*. 2013;200:367-378.
[47] Stalikas, CD. Extraction, separation and detection methods for phenolic acids and flavonoids. *Journal of Separation Science.* 2007;30:3268-3295.
[48] Patel, NS, Hrdlicka, J, Beranek, P, Přibyl, M, Šnita, D, Hammouti, B, Al-Deyab, SS, Salghi, R. Extract of *Phyllanthus fraternus* Leaves as Corrosion Inhibitor for Mild Steel in H_2SO_4 Solutions. *International Journal of Electrochemical Science*. 2014;9:2805-2815.
[49] Benahmed, M, Djeddi, N, Akkal, SH, Laouar S. Saccocalyx satureioides as corrosion inhibitor for carbon steel in acid solution. *International Journal of Industrial Chemisty*. 2016;7:109-120.
[50] Soltani, N, Khayatkashan, M. *Gundelia tournefortii* as a Green Corrosion Inhibitor for Mild Steel in HCl and H_2SO_4 Solutions. *International Journal of Electrochemical Science*. 2015;10:46-62.
[51] Ibrahi, T, Habbab, M. Corrosion Inhibition of Mild Steel in 2M HCl Using Aqueous Extract of Eggplant Peel. *International Journal of Electrochemical Scien*ce. 2011;6:5357-5371.
[52] Hassan, AAM, Abdel-Fatah, HTM. Aqueous Extract of *Salvadora Persica* as a Novel Green Corrosion Inhibitor for Low-Alloy Steel in Acidic Media - Part I. *International Journal of Electrochemical Science*. 2016;11:6959-6975.
[53] Souza, TF, Magalhães, M, Torres, VV, D'Elia, E. Inhibitory Action of *Ilex paraguariensis* Extracts on the Corrosion of Carbon Steel in HCl Solution. International Journal of Electrochemical Science. 2015;10: 22-33.
[54] Loto, CA, Popoola, API. Effect of Cola Acuminata and Camellia Sinensis Mixed Extracts on the Corrosion Inhibition of Mild Steel in 0.5M Sulphuric Acid. *International Journal of Electrochemical Science.* 2012;7:2983-2996.
[55] Eddy, NO, Odoemelam, SA, Ama, IN. Ethanol extract of Ocimum gratissimum as a green corrosion inhibitor for the corrosion of mild steel in H_2SO_4. Green *Chemistry Letters and Review*s. 2010,3:165-172.
[56] Hijazi, KM, Abdel-Gaber, AM, Younes, GO. Electrochemical Corrosion Behavior of Mild Steel in HCl and H_2SO_4 Solutions in Presence of Loquat Leaf Extract. *International Journal of Electrochemical Science*. 2015;10:4366-4380.
[57] Inzunza, RG, Valdez Salas, B, Schorr Wiener, M, Carrillo Beltran, M, Zlatev Koytchev, R, Stoytcheva Stilianova, M, Ramos Irigoyen, R, Vargas Osuna, L, Terrazas Gaynor, J. Aqueous Extract of Creosote Bush (*Larrea tridentata*) Leaves as Green Inhibitor for Carbon Steel in Hydrochloric Acid Solution. *International Journal of Electrochemical Science*. 2013;8:6433-6448.
[58] Venegas, R, Figueredo, F, Carvallo, G, Molinari, A, Vera, R. Evaluation of *Eulychnia acid*a Phil. (Cactaceae) Extracts as Corrosion Inhibitors for Carbon Steel in Acidic Media. *International Journal of Electrochemical Science*. 2016;11:3651-3663.
[59] Ali, IH. Inhibitory Effect of Leaf Extract of Khaya senegalensis (Mahogany) on C-steel Corrosion in 1.0 M Hydrochloric Acid Solution. *International Journal of Electrochemical Scien*ce. 2016;11:2130-2141.
[60] Fouda, AS, Etai, SH, Elnggar, W. Punica Plant extract as Green Corrosion inhibitor for C-steel in Hydrochloric Acid Solutions. International Journal of Electrochemical Science. 2014;9**:** 4866-4883.

[61] Kalla, A, Benahmed, M, Djeddi, N Akkal, S, Laouer, H. Corrosion inhibition of carbon steel in 1 M H_2SO_4 solution by Thapsia villosa extracts. *International Journal of Industrial Chemistry*. 2016; 7:419-429.
[62] Anbarasi, K. Electrochemical and Corrosion Inhibition Studies of *Cucurbita maxima*. *Oriental Journal of Chemistr*y. 2016;32:2139-2145.
[63] Gunavathy, N, Murugavel, SC. Corrosion Inhibition Studies of Mild Steel in Acid Medium Using *Musa Acuminata* Fruit Peel Extract. *E-Journal of Chemistry*. 2012;9:487-495.

Post-harvest quality attributes in carrot produced with organic compost in semi-arid region

A. Figueiredo Neto[1], **R.M. Carneiro**[2] and **F.A.C. Almeida**[3]

[1] Department of Agricultural Engineering, Federal University of San Francisco Valley, office s/n, 48900-300, Brazil
[2] Quality Management Sector, Agricultural Research Company, Brazil
[3] Department of Agricultural Engineering, Federal University of Campina Grande, Ciudad Universitaria, 58000-100, Brazil

The work was developed to evaluate the effect of organic compound in carrots cultivation of the "Brasilia" cultivar and its influence on the post-harvest phase of this vegetable. Experiments were conducted in a communal kitchen garden, cultivated in traditional irrigated farming system, located in the city of Petrolina - PE. The formulation of the organic compound was made of 40% tanned goat manure and 60% waste vegetable residues. The roots were harvested 90 days after sowing, and were soon taken to the postharvest laboratory for analysis. The two treatments for the cultivation of carrot were evaluated as of harvest to check the influence of the compound on the harvested product through moisture analysis, soluble solids (TSS), total titratable acidity (TTA), TSS / TTA ratio, total carotenoid and firmness. Statistical analysis showed a significant difference between the main physical and chemical characteristics of the carrot at 5% between treatments in the various analyzes carried out, indicating that the use of organic compound in the cultivation of carrot is a promising tool in maintaining quality and nutritional content for post-harvest stage.

Keywords: vegetable; post-harvest; nutritional quality

1. Introduction

The use of chemical fertilizer in carrots is an agricultural practice that brings satisfactory results in terms of productivity. However, one should take into account the quality of the final product, since it is known that its disordered use can harm consumers' health, as well as increase production costs and degrade the local environment.

Currently, there are organic fertilizers employed from various sources in the cultivation of vegetables, especially organic compound, which in addition to providing improved physical and chemical properties to the soil, reduces the need for use of mineral fertilizers and also allows vegetable nutritional increase [1].

The carrot (*Daucus carota* L.) is native to Southeast Asia (semi-arid region of Afghanistan), and is a vegetable of the Apiaceae family, of the tuberous roots group, cultivated on large scale in the Southeast, Northeast and South of Brazil [2].

This root responds to organic fertilization especially in low fertility or compacted soils. However, it is essential that the organic fertilizer be well tanned, once it is released on the beds, and then incorporated before planting [3]. The way to minimize the negative effects of these soils which have low fertility would be the use of decomposed or semi-decomposed organic waste [4].

According to [5], vegetables mostly require large amounts of nutrients due to their short cycles. It is known that the use of organic matter positively influences on the germination and rooting of vegetables. The availability of organic compound close to the root system of plants is a desirable feature. The organic matter in the soil stimulates the growth and nutrient uptake by the plant [6].

The beneficial effects of organic waste to the culture of carrot relate to increased organic matter contente in the soil, allowing greater penetration and root distribution, increase in the aggregation index, aeration and infiltration capacity and water storage [7,8].

The choice of organic waste depends on the availability varying between regions and according to the culture that will be used [9]. In the region of Petrolina - PE, goat and sheep breeding has been developing, with the rearing of goats and sheep for the use of milk and meat respectively. With this regional capability, it is possible to obtain the residues of these ruminant animals with ease and quantity, which leads to the search for alternative uses of this organic waste. Among others, it is incorporated and used in ground cover to improve soils cultivated with vegetables. Experimental results showed that for the culture of carrots, the incorporation of 7t ha-1 of poultry litter provided greater leaf mass (45-73 days after sowing) and greater root mass (115 days after sowing) [4].

According to [10], the supply of mineral nutrients, especially those that the soil does not possess in satisfactory conditions during the growing season, and the accumulation of these nutrients by the plant, can influence the quality of the roots in storage. Therefore, when there is a need to opt for post-harvest storage aspect of life, it is convenient to choose carrots grown in soils more nutritionally balanced, harvested at the proper stage of maturity, whole, firm, without insects and microorganisms attack and free of impurities [11].

The existing carotenoids in carrots, responsible for the orange color of the roots, have provitamin A activity, that is, when ingested by humans, they are transformed into vitamin A, constituting one of the main sources of this vitamin for

the population [12]. However, techniques that enable its production without the use of chemicals are needed, in view of product quality. According to [11], the properties that make fruit and vegetables appreciated as food relate to their appearance, taste, odor, texture and nutritional value.

Considering the importance of the theoretical and practical study of quality parameters for the "Brasilia" carrot grown in the region of Petrolina – PE (Brazil), this study aimed to evaluate the effect of organic compost application and its influence on the physical and chemical characteristics in the post-harvest phase of this such appreciated vegetable.

2. Material and Methods

The roots were harvested in an experimental vegetable garden of Farm Project 'Mandacaru', located in the city of Juazeiro-BA, a region of BSwh type climate, according to Koppen classification, having as geographical coordinates 9º24'45.85 "S and 40º30'53.51"O with an altitude of 374m, in the Lower Basin of the São Francisco Valley, a semi-arid region, under traditional irrigated cultivation, following the production phase during the period from March to May 2016. The cultivar used was 'Brasilia' and after 90 days the roots were harvested, and were then stored in the laboratory for proper analysis of their nutritional quality. The data relating to climate variables collected at the meteorological station of Embrapa, during the execution of the experiment are shown in Table 1.

Table 1 Average temperature, relative humidity, precipitation, solar radiation and insolation from March to June 2016.

Month	T (°C)	RH (%)	Pt (mm)	SR (ly/day)	I (hours)
March	24,6	64,93	2	461	7
April	24,3	63,98	2	501	8,1
May	26,4	57,11	0	558	9,5
June	27,7	56,36	0	623	9,7
Average	25,7	60,5	-	535,7	8,5

T = air temperature; RH = relative humidity; Pt = precipitation; SR = solar radiation; I = insolation.

The carrots were cultivated with organic compound (T1) and without organic compound (T2) for subsequent comparison of treatments. The formulation of the organic compound was made of 40% tanned goat manure and 60% vegetable waste residues, which resulted in an organic material which still passed 45 days being revolved and incorporated for proper application in the beds. The physical and chemical characteristics of the soil in the field before the installation of the experiment (Quartzipsamment soil) are in Table 2

Table 2 Chemical and soil particle size characteristics prior to implantation of the experiment at depths of 0-20 and 20-40 cm.

Variable	Soil 0 – 20 cm	Soil 20 – 40 cm
pH	6,9	6,8
	$cmol_c dm^{-3}$	
Ca^{2+}	3,2	2,5
Mg^{2+}	1,8	1,0
Ca + Mg	5,0	3,50
Al^{3+}	0,05	0,05
$H + Al^{3+}$	2,8	2,47
K^+	0,20	0,16
Na	0,04	0,04
SB	5,24	3,7
CEC	8,04	6,17
	$mg\ dm^{-3}$	
P –Melich	29,77	25,66
	%	
OM	0,6	0,44
V	65	60
	$g\ kg^{-1}$	
Clay	7,8	47,4
Silt	80,5	53,5
Sand	911,7	925,0

OM = Organic matter; CTC = Cation exchange capacity [$Ca^{2+} + Mg^{2+} + Na^+ + K^+ + (H^+ + Al^{3+})$]; SB = Sum of bases; V = Base saturation ($Ca^{2+} + Mg^{2+} + Na^+ + K^+$/CTC) x 100

After harvesting, the roots were immediately transported in sanitized cool boxes to the Agricultural Products Storage Laboratory of the Federal University of the São Francisco Valley (UNIVASF) at the Engineering campus in Juazeiro, Bahia, Brazil.

Methods - Physical and Chemical Analysis

After the treatments referring to the use of the compound and its absence, the carrots were evaluated using the following parameters in the post-harvest phase:

Moisture: Moisture content was determined according to the methodology [13] for fruit, adapted to carrot, when approximately 2 g of sample, cut into thin slices and placed in pre-weighed aluminum crucible was weighed. The crucible and sample were placed together in a vacuum oven for a period of 24h at 70 ° C.

Total soluble solids (TSS): The soluble solids content (° Brix) was determined by direct reading on a countertop refractometer, with temperature correction performed by the proposed table from the [14].

Total Titratable Acidity (TTA): determined by titration according to norms of the [14], with results expressed as % of citric acid.

TSS / ATT Ratio: determined by the ratio between the two variables.

Carotene content: The extraction of carotenoids was performed according to the methodology described by [13]. The determination is based on the extraction of the same in hexane and isopropyl alcohol, with subsequent reading in a spectrophotometer at a wavelength of 450 nm.

Total phenolics: these were determined with the use of gallic acid as standard (mg gallic acid equivalents per 100g of carrots on a wet basis [15].

Antioxidant activity: was determined by DPPH (2,2-diphenyl-1-pircril-hydrazyl) method with the use of ethanol extract [16]. The methodologies for the determination were described as consolidated analysis by the Association of Official Analytical Chemists (AOAC) and the Adolfo Lutz Institute.

Firmness: determined with the aid of a countertop penetrometer through two measurements at the equatorial section of the same root, achieving the required pressure for penetration in Newtons [17]. We also used the same procedure for the replicates that were stored for five days to analyze the postharvest life of carrots.

Data were analyzed by ANOVA, evaluated and compared using the SISVAR 4.2 [18], where the experimental design was completely randomized with three replicates for each physical and chemical analysis. Tukey test, considering a 5% significance level was used.

3. Results and Discussion

The average values of physical-chemical analysis of different carrot treatments are shown in Table 3. Moisture is an important factor in the quality of vegetables, since it provides information about its texture, it also increases the economic value since the vegetable has greater mass, so it is remarkable that the carrots treated organically (T1) showed higher values for this feature.

As for the total carotenoid content it is noticeable that the carrots that received the application of organic compost in its cultivation had higher levels of carotenoid (Table 3), confirming the research by [19] in the evaluation of carrot quality in organic crops, which found higher concentrations of carotenoids when compared to those crops with mineral fertilizer.

Table 3 Physical and chemical characteristics of carrots produced with and without organic composto1, Juazeiro - BA, 2016.

Treatments	Variables				
	Humidity %	**Total Carotenoid (mg/100g)**	**Acidity % (AC)**	**TSS (°Brix)**	**TSS/AC**
T1	89,6a	6,54a	0,23a	7,2a	30,6a
T2	87,8b	4,81b	0,24a	6,4b	24,1b
CV%	0,36	2,82	3,50	2,91	3,75

T1 – With organic compound; T2 – No organic compounds. (Averages followed by the same letter in the columns do not differ by Tukey test at 5% probability)

It is found that the use of the compound allows the root of this vegetable to have a good amount of carotenoids, ideal for post-harvest consumption of this product. According to [20], the more intense the color, the higher the beta-carotene content and higher the nutritional value.

The average results obtained for acidity of carrots were expressed as % of citric acid. The organic acids generally decrease after ripening, harvesting and during storage due to oxidation for energy production in the Krebs cycle [21], there was no noticeable difference between treatments in regards to the acidity level of 5% probability.

The values of total soluble solids concentration represent the acids, salts, vitamins, amino acids, some pectins and sugars in vegetables. They are used as an index of total sugars, indicating the degree of maturity [22]. The average results obtained of TSS were expressed in °Brix. After analysis of variance, significant differences were observed between the two carrot treatments, especially higher value for the carrot treated organically.

According [11] TSS / TTA ratio in plants can be regarded as an evaluation criterion of "flavor", and an increase can mean an increase of flavor, and is indicative of the maturity level. In this particular case, there was a significant difference between treatments, and the application of organic compost increased the TSS / TTA ratio, due to the increase in soluble solids, thus indicating an improvement in the organoleptic characteristics of carrots.

Table 4 shows the comparison of the parameters and results of the bioactive compounds in 'Brasília' carrot variety in the two fertilization treatments.

Table 4 Physico-chemical characteristics of carrots produced with and without[1] organic compound, Juazeiro - BA, 2016.

Treatments	Variables			
	Vitamin C (mg/100g)	**Total phenolics (mg/100g)**	**Betacarotene (mg/100g)**	**Antioxidant activity (%)**
T1	22,4a	31,3a	2,20a	42,6a
T2	19,6b	30,5a	1,96a	38,5b
CV%	2,23	1,82	2,35	3,51

[1]T1 – With organic compund; T2 – Without organic compund. (Averages followed by the same letter in the columns do not differ by Tukey test at 5% probability)

The effectiveness of the antioxidant action of bioactive compounds depends on their chemical structure and concentration of these phytochemicals in the food. According to [23] the content of these phytochemicals in vegetables is largely influenced by genetic factors, environmental conditions, in addition to the degree of ripeness, variety of vegetable and growing conditions. It is evident therefore, that the intensity of antioxidant activity of vegetables, especially the 'Brasilia' carrot variety is similar to that reported in other studies. Several factors related to the cultivation of vegetable, like climate and soil conditions, influence the profile of phenolic compounds of vegetables and consequently its antioxidant action.

Physical measurements are highlighted in Table 5. Note that the average weight of carrots treated with the organic compound was statistically higher than those that have not been subjected to organic treatment; this result is important because it demonstrates that it is possible to obtain higher profits since these are sold on weight. However, the length and diameter showed no significant difference between treatments at 5% significance level.

Table 5 Measurements of the physical parameters of carrots with (T1) and without (T2) organic treatment during post-harvest phase of the "Brasilia" carrot.

Treatments	Variables			
	Weight (g)	**Length (mm)**	**Diametre (mm)**	**Firmness (N)**
T1	126,5a	135,7a	33,2a	98,6a
T2	83,8b	137,5a	30,2a	93,8b
CV%	18,77	9,05	10,64	3,11

(Averages followed by the same letter in the columns do not differ by Tukey test at 5% probability)

The firmness of vegetables decreases with maturity and it is a physical characteristic which interferes with consumer acceptability of the roots. This characteristic is obtained by employing resistance gauges or texture, the penetrometer machine being the most used. After compression of the vegetable, a measurement which equals the force needed to overcome the resistance of the plant tissue is obtained [17]. Note that there was no significant difference between treatments at 95% confidence level, and that organically treated carrots had higher stiffness values having thus greater crispness, ie a better texture.

The main variables used to determine the carrot post-harvest quality are firmness, soluble solids, weight loss and the external and internal appearance. But the firmness indicates the direct acceptance of the product by the consumer, weight loss and the carrot's own external appearance provides indication of the potential post-harvest life of this vegetable.

Table 6 states the firmness values of the "Brasilia" carrot over a five days post-harvest life of this vegetable, with no statistical difference in the first two days; however, from the third day there is a statistical difference between treatments. Observations showed that the treatment with organic compound remained at statistically adequate firmness, whereas treatment with no organic compound decreased considerably.

Table 6 Carrot firmness assessment over five days post-harvest life.

Treatment	Days (Firmness N)				
	D1	**D2**	**D3**	**D4**	**D5**
T1	96,8aA	92,3aA	89,1aA	89,0aA	88,8aA
T2	95,2aA	91,4aA	88,4bA	87,8bA	84,0bA
CV% = 3,88					

(Averages followed by the same letters, lowercase and uppercase lines in columns, do not differ by Tukey test at 5% probability).

4. Conclusion

The application of organic compost in the cultivation of "Brasilia" cultivar carrots through the evaluations of moisture, carotenoid content, soluble solids, TSS/AC, weight and strength characteristics showed better quality aspects when compared to carrots which did not undergo organic treatment.

The increase in concentration of soluble solids and the increase of the TSS/ATT ratio in the organic treatment indicates an improvement in the organoleptic characteristics.

The average values of total carotenoids were more significant for carrots treated organically, showing better nutritional characteristics. The significant levels of bioactive compounds for carrots produced with organic compound were significant in the face of those produced with no compound.

Regarding root firmness feature in post-harvest life, the treatment which received application of the organic compound was more effective, making the application of this compound recommendable for this important vegetable.

Acknowledgements The support by National Research Council and UNIVASF is gratefully acknowledged.

References

[1] Souza, P.A.; Negreiros, M.Z.; Menezes, J.B.; Bezerra Neto, F.; Souza, G.L.F.M; Carneiro, C.R.; Queiroga, R.C.F. Características químicas de folhas de alface cultivada sob efeito residual da adubação com composto orgânico. Horticultura Brasileira, Brasília, v.23, n.3, p.699-702, set. 2005.

[2] Filgueira, F.A.R. Novo Manual de Olericultura: Agrotecnologia Moderna na Produção e Comercialização de Hortaliças. 2ª ed. Viçosa – MG, Editora UFV, 2013. 418p.

[3] Emabrapa, Empresa Brasileira de Pesquisa Agropecuária. Cultivo da cenoura (Daucus carota L.), Instrução técnica. In: Centro Nacional de Pesquisa de Hortaliças, Brasília – DF, 2007.

[4] Vieira, M.C. Crescimento e produção de cenoura, considerando cama de aviário semi decomposta, incorporada e em cobertura, em Dourados-MS. SOB Informa, Rio de Janeiro, v.15, n.1, p.17-19, 1996.

[5] Salgado, J.A.A., Almeida, D.L., Guerra, J.G.M., Ribeiro, R.L.D., Sudo, A. Balanço de nutrientes em cultivos de hortaliças sob manejo orgânico. Soropédica: Embrapa Agrobiologia, 9p. 1998.

[6] Negrini, A.C.A., Melo, P.C.T. Efeito de diferentes compostos e dosagens na produção de cenoura (Daucus carota L.) em cultivo orgânico. Revista Brasileira de Agroecologia, v.2, n1., p. 1036-1039, fev. 2007.

[7] Nogueira, F.D. Solo, nutrição e adubação da cenoura e mandioquinha-salsa. Informe Agropecuário, Belo Horizonte – MG, v.10, n.120, p.28-32, 1984.

[8] Araujo, C.; Zárate, N.A.H.; Vieira, M.C. Produção e perda de massa pós-colheita de cenoura "Brasília", considerando doses de fósforo e de cama de frango semi decomposta. Revista Acta Scientiarum Agronomy, Maringá – PR, v.26, n.2, p.131-138, 2004.

[9] Kiehl, E.J. Fertilizantes orgânicos. Piracicaba – SP: Agronômica Ceres, 1985.

[10] Muller, J.J.V. Aspectos do armazenamento de cenouras (Daucus carota L.). In: Muller, J.J.V.; CASALI, V.W.D. Seminários de Olericultura. Viçosa – MG: Imprensa Universitária, v.5, p.01-25, 1982.

[11] Chitarra, M.I.F. & Chitarra, A.B. Pós-colheita de frutas e hortaliças: fisiologia e manuseio, 2 ed. Lavras – MG, Ed. UFLA, 2005. 785 p.il.

[12] Spagnol, W.A.; Park, K.J.; Monteiro, J.M. Taxa de respiração de cenouras minimamente processadas e armazenadas em diferentes temperaturas. Ciência e Tecnologia Alimentos, Campinas, v.26, n.3, p. 552-554, set. 2006.

[13] AOAC. Association of Official Analytical Chemists. Official Methods of Analysis of AOAC international. 16 ed. Maryland: AOAC, 1997, 1141p.

[14] Instituto Adolfo Lutz. Normas analíticas do Instituto Adolfo Lutz: métodos químicos e físicos para análises de alimentos. 3 edição, São Paulo: O Instituto, 1985, v.1, 533p.

[15] Pacheco, P.; Da-Paz, J.G.; Silva, C.O.; Pascoal, G.B. Composição centesimal, compostos bioativos e parâmetros físico-químico do jenipapo (Genipa americana L.) in natura. DEMETRA: Alimentação, Nutrição & Saúde. Vol. 9, p.1041-1054. 2014.

[16] Brand-Wiliams, W.; Cuvelier, M.E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. Food science and Tecnology, v.28, p.25-30, 1995.

[17] Coelho, A.H.R. Qualidade Pós-Colheita de Pêssegos. Informe Agropecuário, v.17, n.180, p.31-39, 1994.

[18] SISVAR 4.2 (Build 39) – Software estatístico. DEU/UFLA. Lavras – MG. Copyrith Daniel Furtado Ferreira 1999-2003.

[19] Evers, A.M. The role of fertilization practices in the yield and quality of carrot (Daucus carota L.). Journal of Agricultura Science in Finland, Helsinki, 61, p.329, 1989.

[20] Filgueira, F.A.R. Novo Manual de Olericultura. 1ª ed. Viçosa – MG, Editora UFV, 2000. 402p.

[21] Fenema, Q. R. Food Chemistry. Marcel Dekker, New York, 991p. 1985.

[22] Bleinroth, E.W. Determinação do ponto de colheita, maturação e conservação das frutas. In: Industrialização de Frutas. Manual Técnico, n.8. Campinas: ITAL, p.1-15, 1991. 206 p.

[23] Melo, E.A.; Maciel, M.I.S.; Lima, V.L.A.; Caetano, A.C.S.; Nascimento, R.J. Capacidade antioxidante de hortaliças usualmente consumidas. Ciência e Tecnologia de Alimentos, v. 26, n.3, p.639-644. 2006.

Probiotics in gastrointestinal-associated diseases

C. Faustino and **L. Pinheiro**

iMed.ULisboa Research Institute for Medicines, Faculty of Pharmacy, Universidade de Lisboa, Avda. Prof. Gama Pinto, 1649-003 Lisbon, Portugal

Disturbance of the bacterial microflora of the gastrointestinal (GI) tract has been associated with the pathophysiology of several GI disorders. Manipulation of the gut microbiota through supplementation with probiotics is an attractive approach to restore, maintain and promote health. Common probiotic formulations for human consumption are mainly based on bacterial strains of *Lactobacillus*, *Bifidobacterium* and the yeast *Saccharomyces boulardii*, either as single or multispecies preparations. Clinical efficacy of a probiotic product is determined by the specific microbial strain, formulation, dose regimen, viability of the microorganisms and time of permanence in the gut. Evidence from research studies and clinical data support the use of probiotics in the prevention and/or treatment of several GI disorders caused either by perturbations in normal gut microbiota, such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), or by pathogenic microorganisms, including GI infections caused by *Helicobacter pylori*, *Clostridium difficile*, and rotavirus. This review discusses the role of probiotics in the prevention and treatment of GI disorders, and proposed mechanisms of action.

Keywords: probiotics; gastrointestinal disorders; inflammatory bowel disease; irritable bowel syndrome; *Helicobacter pylori*; *Clostridium difficile*

1. Introduction

Gastrointestinal (GI) disorders, such as chronic inflammatory GI conditions (e.g., Crohn's disease, ulcerative colitis, irritable bowel syndrome) and acute GI infections are still a major cause of morbidity and mortality worldwide. Many of these diseases, particularly those requiring antibiotic therapy, are becoming increasingly difficult to treat due to increased dissemination of antibiotic resistance among microorganisms and the emergence of (multi)drug-resistant strains [1]. Novel therapeutic approaches are thus essential, and manipulation of the gut microbiota through supplementation with probiotics is an attractive alternative to conventional therapies. Moreover, probiotics are amenable to low-cost and large scale production.

Probiotics have been used in the treatment and prevention of many GI-associated diseases, either alone or as co-adjuvants of standard therapies, with promising results. Probiotic beneficial effects are likely the result of multiple mechanisms of action in the gut that may include effects on host immunity and gut mucosal barrier integrity as well as colonization resistance and production of antimicrobial substances [2]. While some of these mechanisms may be shared among different strains or species, others are specific to certain microorganisms.

Probiotics are associated with a good safety record in the healthy population, primarily related to the use of lactobacilli and bifidobacteria. On the other hand, selection and monitoring of probiotics for individuals compromised gut epithelial integrity and immunocompromised patients demands careful consideration of the risk-benefit ratio.

Translational to the clinical setting requires specification of effective strains or strain combinations and dosage regimen, since therapeutic effects of probiotics are both strain- and dose-dependent [2,3].

2. Mechanisms of Action

Several mechanisms of action are ascribed to probiotics, corresponding to genetic and functional differences in the bacterial strains (even within the same species) [4-6]. A good example is the *Lactobacillus reuteri* strains, present in formulations of probiotics for intestinal applications. While *L. reuteri* SD2112 (ATCC55730) produce the antimicrobial metabolite reuterin that inhibits pathogens in the gut, *L. reuteri* RC-14 health benefits on the intestine are associated to the production of biosurfactants and other antiadhesive constituents as well as to modulating host immunity [4]. Conversely, the same strain might exert its biological effects by multiple mechanisms of action [6]; an example is *L. rhamnosus* GG (LGG) that could reduce inflammation and concomitantly induce adaptive immune cells. Some probiotic strains are capable of producing effects far away from the site of administration. Reasons may lie in the transfer of the organisms from one site to another or in the production of molecules that are adsorbed through the intestine [6]. In general, it is possible to identify a variety of potential mechanisms of action through which probiotics exert their effect on GI-associated disorders [6–15]. Some of the proposed mechanisms, supported by *in vitro* and some *in vivo* experiments, are briefly described.

2.1 Modulation of the host immune system

The intestinal microbiota is fundamental for the maturation of the host immune system and development of immunological resistance. Probiotic bacteria or their metabolites have the capacity to interact with epithelial cells and

immune cells through pattern recognition receptors [5,7,9–12,14,16,17]. Two of these highly specific receptors are Toll-like (microbial-sensing) and NOD (intracellular-sensing) proteins. Toll-like receptors signaling stimulates expression of defensins (small peptides/proteins) in enterocytes [18]. After recognition, immune and epithelial cells produce cytokines that contribute to the control of innate and adaptive immune cells. Lymphocytes (B and T cells) are essential pieces in the adaptive immune response. Many probiotics seem to induce the activation of T helper cells, thus producing cytokines and interfering with immune responses [10].

Immunomodulation by probiotics comprises also the increase of immunoglobulin A (IgA) production by B cells, disabling the proinflammatory response [9,10,14]. Nevertheless, conclusions based on the effect of probiotics on IgA production are still questionable, as some results seem to be contradictory [14]. Although *L. reuteri* led to an increase in proinflammatory cytokines *in vitro*, the same strain revealed an anti-inflammatory activity *in vivo* [7,10].

The immunomodulatory process depends on probiotics survival and permanence in the GI tract, the type of interaction between probiotic and the host immune system, and the strain, dosage and frequency of administration. Immunostimulation of the host seems to be only achieved with a dose of 10^8–10^9 colony forming units (CFU)/d of a strain and a resident time in the intestine between 48 and 72 hours [14].

2.2 Adhesion to intestinal mucosa

Adhesion to intestinal mucosa is the precondition to colonization, being determinant for the probiotic-host interaction, modulation of the immune system, and antagonism against pathogens [9,17].

Intestinal epithelia cells produce the glycoprotein mucin which is the main component of mucus, precluding adhesion of pathogens. Various *Lactobacillus* spp. are reported to adhere to the mucosa, due to mucus adhesion-promoting proteins [5,9,17]. These circumstances might explain an eventual relation between probiotic surface proteins and the concurrent pathogens exclusion from the mucus [9,17]. However, studies on the adhesion-related factors are scarce.

Competitive exclusion of pathogens (where one species of bacteria inhibits or reduces the growth of another species) lies on the competition for nutrients and for mucosal adhesion receptors, being a consequence of the different mechanisms and properties of probiotics [9,14].

2.3 Production of antimicrobial compounds

Considering some GI-related disorders, several authors suggest that probiotics have an impact on microbiota composition positively modifying its qualitative and quantitative profile [14].

Some probiotics can produce antimicrobial compounds which inhibit the growth of bacteria existing in the intestinal lumen [14,17,18]. Lactic and acetic acids have been reported as major antimicrobial compounds of probiotics, due to their high inhibitory activity against Gram-negative bacteria [9,14,17]. The effect on the pathogenic microorganisms may be related to the decrease of intracellular pH or to the intracellular accumulation of the organic acid. Probiotics such as *L. acidophilus* produce lactic acid that, additionally, hamper the adhesion of pathogenic bacteria to epithelial cells and stimulate the clonal expansion of mucosal B lymphocytes to produce IgA [9,14,17].

Along with the aforementioned reuterin (produced by *L. reuteri* SD2112), probiotics may also produce bacteriocins, which are ribosomally synthesized antimicrobial peptides or proteins produced by bacteria, that inhibit the growth of other bacteria reducing the number of pathogens or changing the composition of intestinal microbiota [14,18]. The destruction of pathogen cells results from pore formation and/or inhibition of cell wall synthesis [9]. Some bacteriocins generated by probiotics have been identified. An effective inhibition of foodborne pathogen *Listeria monocytogenes* in mice and pigs was observed for the bacteriocin Abp118, produced by *L. salivarius* UCC118. *Bifidobacterium bifidum* NCFB 1454 produces bifidocin B which inhibits the growth of Gram-positive bacteria [9]. Moreover, probiotic strains can also promote the discharge of defensins by the epithelial cells. Defensins bind to anionic phospholipid groups of the membrane surface through electrostatic interactions, causing the lysis of microorganism cells [7,9,16,18].

Probiotics have also the capacity to deconjugate bile acids [5,6,9,19]. Deconjugated bile acids seems to have a higher antibacterial activity when compared with the ones produced by the host [9].

Several probiotic strains produce metabolites with antifungal properties. In this context, *Lactobacillus* species produce benzoic acid, methylhydantoin, mevalonolactone and short-chain fatty acids (SCFA) [9,17].

2.4 Changes in composition and metabolic activity of gut microbiome

Gut microbiome act as a source of secondary metabolites that are pharmacologically active and able to activate the mammalian liver enzymes [5]. SCFA results from the metabolic activity of intestinal microbiota, and their production seems to be increased by probiotics, which enhances pathogen resistance and stimulates epithelial cells [5,9,14,20]. SCFA have anti-inflammatory properties, being able to enhance epithelial barrier integrity. Production of SCFA also depends on the composition of intestinal microbiota, the diet and the presence of other metabolites [14]. Butyric acid is the most well documented SCFA for colonic epithelial cells, in terms of anti-inflammatory effects [14,21]. Its impact on intestinal function may lie in the inhibition of the activation of nuclear factor kappa B (a prototypical proinflammatory

signaling pathway), in the increased secretion of mucins and antimicrobial peptides, and in the increase of expression of tight junctions proteins [14,20,21].

2.5 Improvement of the intestinal epithelial barrier

Intestinal epithelial cells constitute the surface where immune responses are initiated. A balanced homeostasis of intestinal mucosal function strongly depends on the integrity of the epithelial cells barrier between the intestinal lumen, the lamina propria and the mucosal-associated lymphoid tissue. Microorganisms composing the intestinal microbiota may change the intestinal barrier, making it more or less permeable [14,18]. An increase in mucosal permeability and loss of epithelial integrity are relevant pathophysiological manifestations when considering the GI disorders under analysis. Some species of probiotics show the capacity to diminish the intestinal permeability due to changes in the intracolonic pH, the cellular junction proteins that form the tight junctions and promote colonocytes self-adhesion, and probably in the production of mucins [7,18]. Moreover, defensins also stabilize the gut barrier function [7,16].

3. Therapeutic applications

3.1 Diarrhea treatment and prevention

3.1.1 Treatment of acute infectious diarrhea

Acute infectious diarrhea is a leading cause of mortality worldwide in infants and hospitalized children, especially in developing countries [10]. Most cases of acute, watery diarrhea are due to viral gastroenteritis, which in children are usually caused by rotavirus. On the other hand, traveler's diarrhea and foodborne diarrheas are commonly of bacterial origin (e.g., enterotoxigenic *Escherichia coli* (ETEC), *Campylobacter*, *Shigella*, or *Salmonella*) [22].

Probiotics have been extensively studied in the treatment and prevention of acute diarrheal states, especially in the pediatric population. Most of the studies involved lactobacilli strains (mainly *Lactobacillus rhamnosus* GG, *L. reuteri*, *L. casei* and *L. acidophilus*), *Bifidobacterium lactis*, *Streptococcus thermophilus* and the yeast *Saccharomyces boulardii*, used either alone or in combination [2,23].

Data from several placebo-controlled, randomized clinical trials (RCTs) point to a statistically significant benefit in the use of probiotics, mostly LGG and *S. boulardii*, for the treatment of acute infectious diarrhea [2,3,19,23–27]. The effect of probiotics is both strain-dependent and dose-dependent, with doses higher than 10^{10}–10^{11} CFU/d usually achieving better results [23,24,28]. Fang et al. [29] studied the dose-dependent effect of *L. casei rhamnosus* Lcr35 in children with acute rotaviral gastroenteritis and found that a minimal effective dose of 6×10^{8} CFU daily, for 3 days, was necessary for quantitative reduction of fecal rotavirus shedding in pediatric patients [29].

Probiotics seem to be more effective in the treatment of acute watery diarrhea, particularly if caused by rotavirus, than in invasive bacterial diarrhea, and achieve better therapeutic effect when administered early in the course of the diarrheal state [23,24]. The efficacy of a probiotic formulation combining *Bacillus mesentericus*, *Clostridium butyricum* and *Enterococcus faecalis* in the treatment of acute infectious diarrhea in hospitalized children with *Salmonella* and rotavirus gastroenteritis revealed significant reduction in the severity of symptoms for rotavirus-infected children only [30].

The available body of evidence supports recent guidelines from both the European Society for Pediatric Infectious Diseases (ESPID) and the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) that recommend specific probiotics with proven clinical efficacy, namely LGG and *S. boulardii* CNCM I-745, as an adjunct to rehydration therapy for the management of acute gastroenteritis in children [31].

Probiotics have been found to shorten the duration of acute infectious diarrhea by approximately 1 day in adults and children, and reduce stool frequency on day 2, according to a Cochrane review involving 63 studies with a total of more than 8,000 participants, mainly receiving LGG, *S. boulardii* or *Enterococcus faecium* SF68 [2]. However, *E. faecium* SF68 is a possible recipient of the vancomycin-resistance genes and it is not advisable in the management of children with acute gastroenteritis [2]. The probiotic formulation VSL#3, a mixture of 8 probiotic strains (*L. acidophilus*, *L. paracasei*, *L. bulgaricus*, *L. plantarum*, *B. breve*, *B. infantis*, *B. longum* and *S. thermophilus*) is preferable for the pediatric treatment of acute rotavirus diarrhea. Oral administration of VSL#3 during 4 days to infected children significantly reduced stool frequency and the requirement for oral rehydration salts compared to the placebo without showing side effects, resulting in better recovery rates [1,32].

3.1.2 Prevention of acute infectious diarrhea

There is evidence that certain probiotics can prevent acute diarrheal states, both in adult and children populations. A meta-analysis of 34 placebo-controlled, RCTs evaluating the efficacy of probiotics in preventing acute diarrhea revealed a reduction of 35% with substantial heterogeneity [33]. The effect was found to depend on the probiotic strain and on the age of the host, with probiotics reducing the risk of acute diarrhea in children by 57% but only by 26% in adults [33].

In a double-blind RCT, oral administration of LGG (6×10^9 CFU/twice daily) to 81 children hospitalized for non-diarrheal complaints significantly reduced the risk of nosocomial diarrhea in comparison with placebo, particularly nosocomial rotaviral gastroenteritis [27,34]. In another study, supplementation of an infant formula with *B. bifidum* and *S. thermophilus* reduced the incidence of acute diarrhea and rotavirus shedding in infants admitted to hospital [10,27].

On the other hand, administration of *L. reuteri* DSM 17938 did not significantly reduce the risk of nosocomial diarrhea or rotavirus gastroenteritis in hospitalized children [35]. However, daily administration of *L. reuteri* DSM 17938 (10^8 CFU/d) for 3 months to healthy children attending day care centers reduced the frequency and duration of diarrheal episodes compared to placebo [36]. Moreover, a study conducted in a community setting indicates that daily intake of a probiotic drink containing *L. casei* strain Shirota for 12 weeks reduced the occurrence of acute diarrhea in young children compared to a non-supplemented nutrient drink [37].

Regarding the prophylaxis of traveler's diarrhea (TD), which is a common health problem when travelling to Africa, South East Asia, or Central and South America, a meta-analysis of 12 RCTs by McFarland [22] concluded that probiotics, namely *S. boulardii* and a mixture of *L. acidophilus* and *B. bifidum*, are safe and effective for this purpose [22]. Probiotic use was associated with a significant reduced risk of 85% in developing TD [38]. The efficacy of the treatment depended on duration, dosage and type of probiotic, traveler compliance, travel destination and probiotic viability during the trip. Further studies are needed before probiotics can be considered as an alternative option to antibiotic therapy for the prophylaxis of TD.

3.1.3 Prevention of antibiotic-associated diarrhea

Diarrhea is often a side effect of antibiotic therapy due to disturbance of the normal host GI microflora enhancing overgrowth of enteropathogens, such as *Clostridium difficile*, responsible for diarrhea and colonic inflammation (colitis) [1]. Prevention of antibiotic-associated diarrhea (AAD) by probiotics may be mediated by several mechanisms, such as local competition for adhesion receptors and nutrients, production of antimicrobial substances, and stimulation of intestinal antigen and nonspecific immune responses that enhance restoration of the gut microflora [39].

One third of the patients on antibiotic therapy commonly develop AAD at any point from the initiation of the treatment up to several weeks after its discontinuation [39,40]. Several factors can influence the development of AAD, such as the type of antibiotic, age and health conditions of the host, etiology and hospitalization status. A higher risk of AAD is commonly linked to antibiotics that act on anaerobes, including broad-spectrum penicillins, cephalosporins, clindamycin and fluoroquinolones [41–43].

Several meta-analysis and systematic reviews indicate a statistically significant association between probiotic administration and reduction of AAD, with the most effective strains being LGG and *S. boulardii* [23,38–42]. These probiotics are actually recommended for the prevention of AAD in children by the ESPGHAN working group on probiotics [43].

L. reuteri ATCC 55730 has also been shown to reduce the incidence of AAD in adults in a hospital setting [44], however this strain carries transferable resistance traits for tetracycline and lincomycin, and should preferably be replaced by *L. reuteri* DSM 17938, with no unwanted plasmid-borne resistances [26]. A RCT to assess the efficacy of *L. reuteri* DSM 17938 for the prevention of diarrhea and AAD in children (NCT02871908) is currently recruiting [45].

Since the beneficial effects of probiotics are strain-specific, some meta-analysis have been performed on the efficacy and safety of only one type of microorganism in order to avoid heterogeneity from pooling data on different strains [38,41,42]. A systematic review with meta-analysis of *S. boulardii* for the prevention of AAD in children and adults showed that 1 in 10 cases of AAD could be prevented by co-administration of the probiotic [41].

The dose-response effect of probiotics on the incidence of AAD has also been evaluated. In hospitalized adult patients on antibiotic therapy, daily administration of a probiotic mixture (*L. acidophilus* NCFM, *L. paracasei* Lpc-37, *B. lactis* Bi-07 and Bi-04) up to 7 days after the end of the antibiotic course showed a significant dose-response effect on AAD, with incidences of 12.5% in patients on the high-dose regimen (1.70×10^{10} CFU/d) compared with 19.5% in the low-dose regimen (4.17×10^9 CFU/d) group and 24.6% in the placebo group [46].

It is worth mention that in most of these studies, the type and/or dose of the antibiotic have not been randomized along with the probiotic arm. In healthy volunteers taking amoxicillin (500 mg twice daily for 7 days) and 5 g of a multispecies probiotic preparation (10^9 CFU/g twice daily for 15 days) containing *B. bifidum* W23, *B. lactis* W18, *B. longum* W51, *E. faecium* W54, *L. acidophilus* W37 and W55, *L. paracasei* W72, *L. plantarum* W62, *L. rhamnosus* W71, and *L. salivarius* W24, changes in microbial composition and metabolic activity of the intestinal microbiota over time suggested that the amoxicillin effect was modulated by probiotic intake [39].

3.1.4 *Clostridium difficile*-associated diarrhea

Clostridium difficile infection (CDI) is a common complication of antibiotic therapy, with symptoms ranging from mild diarrhea and colonic inflammation to severe pseudomembranous enterocolitis, which can lead to sepsis and death [47,48]. *Clostridium difficile*-associated diarrhea (CDAD) is most frequent in elderly and hospitalized patients receiving broad-spectrum antibiotics (e.g., penicillins, cephalosporins, clindamycin and fluoroquinolones) but other risk factors include the use of proton-pump inhibitors, H_2-antagonists, methotrexate, and existence of other GI pathologies

associated with impaired mucosal barrier function [49]. Metronidazole and vancomycin are the standard therapeutic regimens for CDI, however recurrence and relapse have been observed, even after repeated antibiotic treatments [47, 49].

Probiotics can prevent gut colonization by *C. difficile* and are thus promising agents for the prophylaxis of CDAD. Moreover, certain strains of lactobacilli produce peptide metabolites with lytic activity against several *C. difficile* strains while *S. boulardii* produces a serine protease that degrades *C. difficile* toxins A and B [38,50]. Co-treatment with probiotic *S. boulardi* is associated with a significant decrease in the risk of CDI recurrence [38,41].

Fecal sample analysis of *C. difficile* toxins in hospitalized elderly patients on antibiotic therapy revealed that 46% of the patients who received co-treatment with a probiotic mixture containing *L. acidophilus* and *B. bifidum* were toxin-positive compared with 78% in the placebo group [47–49]. Consumption of cheese supplemented with both *L. rhamnosus* HN001 and *L. acidophilus* NCFM by healthy, elderly volunteers was also associated with a trend towards lower fecal counts of *C. difficile* compared with the non-supplemented cheese for the same period [51].

Co-administration of a probiotic drink containing *L. casei*, *L. bulgaricus* and *S. thermophilus* to hospitalized elderly patients (mean age 74 years old) on antibiotic therapy lowered the incidence of CDAD [47–49]. Moreover, a dose-response efficacy study with a probiotic formulation containing *L. acidophilus* CL1285 and *L. casei* LBC80R (50 billion CFU/capsule) for CDAD prophylaxis showed that high doses (2 capsules/d) were more efficient than low doses (1 capsule/d) in reducing the incidence of CDAD in hospitalized adult patients [47–49,52].

Contradictory results have been reported on the efficacy of probiotics for the prevention of recurrent CDI, which may be due to differences in the study population, type and dose of probiotic, and/or duration of the treatment [53]. A recent meta-analysis and a Cochrane review have found that probiotics significantly reduce the risk of CDAD by approximately 60% [48,49] but not the incidence of CDI [48].

On the other hand, lower CDI rates have been found among antibiotic-treated patients taking probiotics during time at risk when compared with placebo, and results from a meta-analysis suggest that primary prevention of CDI can be achieved with specific probiotic agents, the most promising ones being *S. boulardii* and the probiotic mixture *L. acidophilus* CL1285 and *L. casei* LBC80R [53]. A probiotic combination of *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 containing 50 billion live bacteria, commercially available as both fermented beverages and targeted-release capsules with an enteric coating, has been associated with decreased incidences of CDI in human clinical trials [54]. The commercial products, as well as the individual strains, have shown antimicrobial activity against *C. difficile* and ability to neutralize toxins A and B *in vitro* [54].

Overall, evidence from systematic reviews and meta-analysis suggest a beneficial effect of probiotics as adjuvant therapy in preventing primary CDI, but there is insufficient data to support their use in secondary prevention of recurrent CDI, requiring larger studies for further evaluation [53,54]. Moreover, antibiotic regimens need to be specified in order to determine the influence of antibiotic class or type, dose and duration of treatment in probiotic efficacy [53,55].

3.2 Helicobacter pylori eradication

Helicobacter pylori are a group of highly prevalent human pathogens infecting approximately half of the world population [56]. *H. pylori* infection has been linked to dyspepsia, chronic gastritis, peptic ulcer, and gastric cancer [56]. Current treatment guidelines recommend concomitant triple therapy for 7-14 days combining a proton pump inhibitor (PPI) with the antibiotics clarithromycin and either amoxicillin or metronidazole as the first approach for *H. pylori* eradication [56,57]. However, eradication rates have declined from 90% to 70% with this treatment, mainly due to increase in antibiotic resistance and poor patient compliance resulting from frequent adverse effects associated with antibiotic therapy, such as diarrhea, headache, loss of appetite, nausea, abdominal pain and skin rash [56,57].

In order to overcome antibiotic resistance, levofloxacin-containing triple therapy as well as sequential and concomitant quadruple therapies with addition of either a bismuth salt or another antibiotic have been developed as second line treatments for *H. pylori* eradication, with quadruple regimens being associated with higher incidence of adverse side effects [56,57].

Probiotics can prevent GI-related side effects of antibiotic therapy, particularly AAD, by aiding in the restoration of gut microbiota. Moreover, probiotics such as *Lactobacillus*, *Bifidobacterium* and *S. boulardii* have demonstrated anti-*H. pylori* activity both *in vitro* and in animal models of *H. pylori* infection [17,56].

The *H. pylori* inhibitory effect of probiotics may be due to competition with the pathogen for adhesion sites, production of antimicrobial metabolites (e.g., *L. johnsonii* La1, *L. casei* strain Shirota, *L. lactis*, *Bacillus subtilis*) or by enhancing mucin secretion (e.g., *L. plantarum* 299v and LGG). Lactic acid production by lactic acid bacteria (LAB) and bifidobacteria decreases pH, which attenuates the hypochlorhydria associated with *H. pylori* infection and can also inhibit *H. pylori* urease [17,56]. Moreover, probiotics show immunomodulatory effects that reduce *H. pylori*-induced gastric activity and inflammation by controlling the balance of proinflammatory and anti-inflammatory cytokines [17,56]. A decrease in specific IgG antibodies to *H. pylori* infection parallel to a reduction of gastric inflammation has been observed in animal models following probiotic intake, with simultaneous enhancement of secretory IgA production in the intestinal epithelium and strengthening of the mucosal barrier [17,56].

Probiotic monotherapy using strains such as *L. johnsonii* La1, *L. casei* strain Shirota, *L. gasseri* OLL2716, *L. reuteri* ATCC 55730, *L. acidophilus* La5 or *B. bifidum* BF-1 has been shown to decrease bacterial load in humans, but probiotics alone are not able to eradicate *H. pylori* [17,57]. On the other hand, several systematic reviews and meta-analysis suggest that adjuvant therapy with probiotic *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, and their blends, can significantly increase eradication rates in comparison with placebo or no additional treatment while simultaneous reducing antibiotic-related side effects (particularly AAD) and improving patient compliance [41,56,58–60]. Sub-group analysis concerning data from 33 RCTs involving a total of 4,459 patients showed that the impact of probiotic supplementation on the efficacy of *H. pylori* eradication therapy was statistically significant only for some individual strains: *L. acidophilus*, *L. casei* DN-114001, *L. gasseri*, and *B. infantis* 20136 [58].

H. pylori eradication rates improved from 68.9% to 83% after addition of the probiotic *B. infantis* 2036 to standard triple therapy [56,58]. On the other hand, probiotic pretreatment for 2 weeks before addition to either triple therapy or sequential therapy increased eradication rates up to 90% [56,58]. Furthermore, a 4-week treatment with *L. gasseri* OLL2716-containing yogurt twice daily 3 weeks prior first-line *H. pylori* eradication therapy, improved eradication rate of primary clarithromycin-resistant *H. pylori* strains (compared to triple therapy alone), in agreement with previous *in vitro* studies showing the ability of *L. gasseri* OLL2716 to suppress both clarithromycin-susceptible and -resistant *H. pylori* strains [56,58].

A 2-week supplementation with 100 mL of fermented milk containing probiotic *L. casei* DN-114 001 enhanced the therapeutic benefit of standard triple therapy in *H. pylori*-positive children with gastritis [56,58]. Kefir, a fermented milk drink containing *Lactobacillus*, *Lactococcus* and yeast, has also been found to increase eradication rate in dyspepsia patients on standard triple therapy when compared with placebo-containing milk [56,58].

Other studies support the beneficial effect of *L. reuteri* strains (known to secrete the antimicrobial metabolite reuterin that inhibits *H. pylori* growth *in vitro*), which have been associated with increased eradication rates, decreased serum gastrin-17 levels in *H. pylori* asymptomatic patients, and decreased incidence of adverse events both in first-line triple therapy and in second-line triple therapy with levofloxacin [56,58].

Regarding probiotic blends as adjuvants in *H. pylori* eradication therapy, a recent systematic review and meta-analysis [60] indicate that the most effective multi-strain probiotics are a combination of *L. acidophilus* and *B. animalis*, and an eight-strain mixture composed of *L. acidophilus*, *L. casei rhamnosus*, *L. plantarum*, *L. reuteri*, *L. salivarius*, *L. sporogenes*, *B. infantis*, and *B. longum* [60].

Overall, the benefits conferred by probiotics in coadjuvant therapy for *H. pylori* eradication are strain-specific and influenced by the chosen antibiotic regimen, being more significant with relatively ineffective antibiotic regimens [56,58,59].

3.3 Inflammatory bowel disease

Inflammatory bowel disease (IBD) comprises idiopathic chronic relapsing inflammatory disorders of the GI tract, such as Crohn's disease (CD) and ulcerative colitis (UC). In the former, inflammation is often discontinuous, transmural and may involve any part of the GI tract (although predominantly affecting the terminal ileum and colon) while in the latter inflammation is typically continuous with mucosal lesions confined to the colon [18,61]. The pathophysiology of IBD is multifactorial and still unclear, linked to an abnormal intestinal immune response to the gut microbiota in genetically susceptible individuals [18,61].

Microbiota changes in IBD are characterized by a higher proportion of *Actinobacteria* and *Enterobacteria* (containing many of the gut pathogens) and decreased load of *Bacterioidetes* and *Firmicutes*, especially in regions of active inflammation [20,61]. The latter groups of bacteria are known to produce SCFA metabolites with potent anti-inflammatory activity [20,61].

Anti-inflammatory drugs (topical or systemic), such as aminosalicylates (e.g., 5-aminosalicylic acid (mesalamine) and its azo derivatives sulfasalazine, balsalazide, and olsalazine) and corticosteroids (e.g., prednisone, hydrocortisone) are the first approach in the treatment of IBD. In case of persistent disease activity, adverse events to aminosalicylates or severe refractory IBD, immunosuppressant drugs are used, such as thiopurines (azathioprine or 6-mercaptopurine), cyclosporine or tacrolimus, and ultimately biological agents like infliximab (a tumor necrosis factor (TNF)-α inhibitor), methotrexate, or natalizumab and vedolizumab (integrin inhibitors) [20,61,62].

Probiotics are promising agents for treating and preventing relapse of IBD due to their impact on host gut microbiota and mucosal immunoregulation via restoration of the balance between proinflammatory and anti-inflammatory cytokines [18,61]. Several RCTs showed benefits of a range of probiotics in IBD, particularly UC and pouchitis, while current evidence in CD is less promising [18,61].

3.3.1 Crohn's disease

In patients with CD, multiple studies comparing probiotics and placebo showed no significant difference in clinical outcomes [18,20,61], for instance, none of the *Lactobacillus* strains co-administered with steroids to adult CD patients showed any effect on endoscopic remission or on the Crohn's disease activity index (CDAI) scores [61]. The commensal bacterium *Faecalibacterium prausnitzii* found in lower numbers in patients with CD has been shown to

reduce proinflammatory cytokines in Caco-2 cell lines and to attenuate the severity of induced colitis in mice [3,61] with potential for the treatment of CD.

Small-sized studies showed a clinical benefit of *S. boulardii* in decreasing stool frequency and improving CDAI scores, accompanied by a decrease in the rate of clinical relapse as measured by the CDAI [61,63]. For CD patients in remission treated with the yeast, the lactulose/mannitol ratio used to measure intestinal permeability decreased in the probiotic group compared with placebo, suggesting a beneficial effect [61,63]. However, a larger trial failed to provide any clinical benefit of *S. boulardii* in relapse rates for CD patients in remission after salicylate or steroid therapies, according to the CDAI score [61,63].

Very recently, the probiotic mixture VSL#3 has been evaluated as an alternative to ineffective single-strain probiotic formulations in the prevention of CD recurrence after surgery [63,64]. Patients with CD were given either placebo or 1 sachet of VSL#3 (900 billion viable bacteria) within 30 days of ileocolonic resection and re-anastomosis. After 3 months, patients receiving VSL#3 had reduced mucosal levels of inflammatory cytokines compared with those receiving placebo, although there were no statistical differences in endoscopic recurrence between the two groups. However, VSL#3 was found to exert 12-month protective benefit in patients that started therapy immediately after surgery compared with those starting it after 3 months of placebo treatment, which warrants further investigation of VSL#3 as a potential probiotic agent for CD [63,64].

Altogether, there is not enough evidence for a beneficial role of probiotics in induction or maintenance of remission in CD [3,61,63].

3.3.2 Ulcerative colitis

Probiotics, either alone or in addition to standard IBD therapy, have shown promising results in induction and maintenance of remission of UC.

Bifidobacteria-fermented milk, containing *Bifidobacterium* strains (*B. breve* and *B. bifidum*) combined with *L. acidophilus*, showed significant improvement in cytokine profile, Clinical Activity Index (CAI) and histological scores when compared with placebo or no additional treatment [61]. On the other hand, a triple combination of *B. longum*, *L. acidophilus* and *E. faecalis* improved clinical symptoms and colonic mucosal inflammation in UC patients when combined with 5-aminosalicylic acid [61].

On the other hand, the non-pathogenic strain *E. coli* Nissle 1917 was found to be equivalent to mesalamine for UC remission and maintenance, both in adults and in children [20]. Similarly, no difference in preventing relapse of UC has been found between the probiotic (10^{11} CFU/d) and standard mesalamine therapy (2.4 g/d) when combined with prednisone and oral gentamicin [20].

Ambulatory adult patients and children with mild to moderate UC not responding to conventional therapy showed induction of remission after treatment with the multistrain probiotic VSL#3, without adverse events [20]. Regarding the effect of VSL#3 in the pediatric population, administration of the probiotic mixture to children with acute UC, along with concomitant steroid induction and mesalamine maintenance therapy, led to remission in 92.8% of treated patients compared with only 36.4% in the placebo group, with relapse rates of 21.4% and 73.3%, respectively, within 1 year of follow-up [20,61]. Overall, probiotics may be beneficial in mild-to-moderate UC as adjuvant therapy [3,20].

3.3.3 Pouchitis

Pouchitis is an iatrogenic condition that occurs in approximately 50% of patients following proctocolectomy with ileal pouch-anal anastomosis for chronic UC. Broad-spectrum antibiotics, such as ciprofloxacin and metronidazole, are the standard therapy for pouchitis [61,65].

According to a recent Cochrane review, no difference was observed in clinical improvement between LGG and placebo for the treatment of acute pouchitis, neither between *B. longum* and placebo for prevention of pouchitis [65]. On the other hand, a pooled analysis suggested that VSL#3 was more effective than placebo for maintenance of clinical remission in chronic pouchitis [3,65]. Moreover, 90% of patients on the VSL#3 probiotic group had no episodes of acute pouchitis during the 12-month follow-up compared to 60% in the placebo group, suggesting a preventive role for the probiotic [3,65].

Evidence from systematic reviews and meta-analysis suggests that probiotics (particularly VSL#3) can provide considerable benefit in the treatment of acute pouchitis and maintenance of clinical remission [61,65].

3.4 Irritable bowel syndrome

Irritable bowel syndrome (IBS) is a GI disorder characterized by the association between recurrent abdominal pain or discomfort and a change in stool consistency or frequency [66] whose etiology remains unclear. The pathophysiology of IBS is multifactorial and has been linked to alterations of the gut microbiota, small-bowel bacterial overgrowth, and abnormal function along the brain-gut axis associated with GI hypersensitivity, motor dysfunction, and deregulated mucosal immune responses directly related to deterioration of intestinal barrier function, leading to disease symptoms like discomfort and pain, diarrhea, constipation, or alternating bowel movements [12,66].

There is no cure for IBS and treatment focuses on the relief of symptoms. Changes in diet and lifestyle are often sufficient to control mild IBS but moderate and severe IBS may require medication. Fiber supplements are recommended for constipation and anti-diarrheal drugs for the control of diarrhea, while anticholinergic and antispasmodic drugs can help relieve painful bowel spasms. Antidepressant drugs have also been used when IBS symptoms include abdominal pain or depression. Antibiotics are used in IBS cases associated with intestinal bacterial overgrowth [66]. Most of these drugs have been associated with adverse events, such as diarrhea, constipation and bloating.

Probiotics are likely to play a role in amelioration of IBS symptoms due to their impact on the gut microbiota [21]. Furthermore, several studies showed a luminal dysbiosis in IBS patients characterized by decreased lactobacilli and bifidobacteria, the genera frequently used in probiotic products, which can help replenish these species [66]. Probiotics can exert an additional beneficial effect on the intestinal mucosa through suppression of the growth and binding of pathogenic bacteria, modulation of the host immune response and enhancement of mucosal integrity, with improvement of bowel dysmotility [21]. Results from *in vitro* studies and from *ex vivo* cell cultures, as well as from several animal models (mice and rats) in which the epithelial barrier integrity had been disrupted before administration of probiotics, showed alleviation of IBS-like symptoms and restoration of the barrier function [21].

A systematic review of 19 RCTs with a total of 1,650 IBS patients showed that probiotics were significantly superior to placebo in the relief of IBS symptoms and improvement of quality of life (QoL) but failed to identify the most beneficial species and strain [67]. A more recent meta-analysis [68] confirmed the beneficial effects of probiotics, namely statistically significant improvements in terms of global symptom, abdominal pain, bloating and flatulence scores. *L. plantarum* 299v (DSM 9843) or probiotic combinations were the most effective [68–70].

The dosage in probiotic formulations is also relevant for the therapeutic effect. In a clinical trial conducted with 362 women with IBS treated with either placebo or probiotic capsules containing *B. infantis* 35624 at a dose of 10^6, 10^8 or 10^{10} CFU/capsule for 4 weeks, only the 10^8 CFU dosage was superior to placebo in the relief of IBS symptoms, particularly pain and discomfort [68,71]. The higher dosage (10^{10} CFU) was associated with low bioavailability due to formulation problems, namely resistance to dissolution [68,71]. On the other hand, this same dosage had proven effective when provided in a milk-based formulation to IBS patients, where the beneficial effects of *B. infantis* 35624 were associated with restoration of the balance between proinflammatory and anti-inflammatory cytokines [68,71].

Overall, probiotics are effective in amelioration of IBS symptomatology, showing both strain-specific and dose-dependent effects.

4. Safety of probiotics

Probiotics are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA). Traditional LAB, which are normal colonizers of the human body and prevalent in fermented food, have a safety record of human consumption. Bifidobacteria share a similar safety profile [19]. However, the therapeutic use of probiotics is recommended only in specific situations and for certain probiotic strains at appropriate doses.

Probiotics are usually intended for the otherwise generally healthy population and several studies have documented the safety of short-term probiotic interventions in immunocompetent individuals [39,72]. Moreover, no increased incidence of lactobacilli-induced bacteremia has been observed after the introduction and widespread use of dairy products supplemented with probiotic *Lactobacillus* in Northern European countries, according to population-based studies [53].

However, probiotic use by preterm infants, immunocompromised and chronically ill individuals requires caution and the risk-benefit ratio must be carefully considered before recommendation [73]. There are also several underlying risk factors, such as indwelling catheters and GI disorders associated with compromised gut permeability and/or immunity that can enhance bacterial/fungal translocation and predispose to probiotic sepsis [40].

There is a risk of fungemia with *S. boulardii* in immunocompromised individuals and sporadic infections, including bacteremia, septicemia, pneumonia and deep abdominal abscesses, have been reported in neonates, severely debilitated and immunocompromised patients, such as those with HIV, severe neutropenia, and cancer, as a result of probiotic administration [74]. On the other hand, there are studies reporting the safety of probiotics in preterm neonates, and in immunocompromised children and adults with HIV [38]. Endocarditis has been reported in patients with damaged or artificial heart valves treated with lactobacilli [74]. There are also documented cases of adverse events that resulted from probiotic use by patients under enteral or parenteral nutrition with pancreatitis or undergoing transplant [72]. Therefore, it is advisable to avoid probiotic use in populations at risk for adverse events until further trials that produce robust data on probiotic safety have been conducted.

5. Conclusions

Current evidence supports the role of probiotics in a broad range of GI disorders, the best documented case being the prevention and treatment of acute infectious diarrhea. Future studies addressing safety, identity, stability and GI

survival of probiotics are needed to identify the optimum strain(s) or strain combinations for specific GI disorders, optimum doses and duration of therapy. Moreover, mechanisms of action should be clarified before translational to the clinical setting.

The clinical efficacy of a probiotic product depends on the specific strain, dosage, formulation, residence time in the gut, microbial viability on the shelf and in the intestine. Thus, it is difficult to make recommendations concerning specific probiotic products unless clinically tested in its final formulation and marketed dosage. Additionally, the quality, composition, and formulation among probiotic products vary widely, mainly due to lack of regulation of the probiotics market since mostly all probiotic products are marketed as foods or dietary supplements. Probiotics are mainly targeted to the generally healthy population and although empirical evidence suggests there is minimal potential for harm with probiotics, due caution is recommended in the administration of probiotics to immunocompromised individuals.

References

[1] Culligan EP, Hill C, Sleator RD. Probiotics and gastrointestinal disease: successes, problems and future prospects. Gut Pathogens 2009; 1:19.

[2] Allen SJ, Martinez EG, Gregorio GV, Dans LF. Probiotics for treating acute infectious diarrhea. Cochrane Database of Systematic Reviews 2010; CD003048.

[3] Ringel Y, Quigley EMM, Lin HC. Using probiotics in gastrointestinal disorders. American Journal of Gastroenterology 2012; 1:34-40.

[4] Cadieux P, Wind A, Sommer P, Schaefer L, Crowley K, Britton RA, et al. Evaluation of reuterin production in urogenital probiotic *Lactobacillus reuteri* RC-14. Applied and Environmental Microbiology 2008;74:4645-49.

[5] Rupa P, Mine Y. Recent Advances in the Role of Probiotics in Human Inflammation and Gut Health. Journal of Agricultural and Food Chemistry. 2012; 60:8249-56.

[6] Reid G. Probiotics: definition, scope and mechanisms of action. Best Practice & Research Clinical Gastroenterology. 2016; 30: 17-25.

[7] Lee BJ, Bak Y-T. Irritable Bowel Syndrome, Gut Microbiota and Probiotics. Journal of Neurogastroenterology and Motility. 2011; 17:252-66.

[8] Vitetta L, Briskey D, Hayes E, Shing C, Peake J. A review of the pharmacobiotic regulation of gastrointestinal inflammation by probiotics, commensal bacteria and prebiotics. Inflammopharmacology. 2012; 20: 251-66.

[9] Bermudez-Brito M, Plaza-Díaz J, Muñoz-Quezada S, Gómez-Llorente C, Gil A. Probiotic Mechanisms of Action. Annals of Nutrition and Metabolism. 2012; 61: 160-74.

[10] Saad N, Delattre C, Urdaci M, Schmitter JM, Bressollier P. An overview of the last advances in probiotic and prebiotic field. Food Science and Technology 2013; 50:1-16.

[11] Kim JW. What Is the Therapeutic Mechanism of the Probiotics in Irritable Bowel Syndrome Patients With Visceral Hypersensitivity? Journal of Neurogastroenterology and Motility. 2014; 20:555-557.

[12] Vitteta L, Briskley D, Alford H, Hall S, Coulson S. Probiotics, prebiotics and the gastrointestinal tract in health and disease. Inflammopharmacology 2014; 22:135-54.

[13] Vitetta L, Bambling M, Alford H. The gastrointestinal tract microbiome, probiotics, and mood. Inflammopharmacology. 2014; 22:333–39.

[14] Reis SA, Conceição LL, Siqueira NP, Rosa DD, Silva LL, Peluzio MCG. Review of the mechanisms of probiotic actions in the prevention of colorectal cancer. Nutrition Research. 2017; 37: 1-19.

[15] Dimidi E, Christodoulide S, Scott M, Whelan K. Mechanisms of Action of Probiotics and the Gastrointestinal Microbiota on Gut Motility and Constipation. Advances in Nutrition. 2017; 8: 484-94.

[16] Comito D, Cascio A, Romano C. Microbiota biodiversity in inflammatory bowel Disease. Italian Journal of Pediatrics. 2014; 40:32

[17] Boltin D. Probiotics in *Helicobacter pylori*-induced peptic ulcer disease. Best Practice & Research Clinical Gastroenterology 2016; 30:99-109.

[18] Bron PA, Kleerebezem M, Brummer R-J, Cani PD, Mercenier A, MacDonald TT, Garcia-Ródenas CL, Wells JM. Can probiotics modulate human disease by impacting intestinal barrier function? British Journal of Nutrition 2017; 117:93-107.

[19] Verna EC, Lucak S. Use of probiotics in gastrointestinal disorders: what to recommend? Therapeutic Advances in Gastroenterology 2010; 3:307-19.

[20] Derikx LAAP, Dieleman LA, Hoentjen F. Probiotics and prebiotics in ulcerative colitis. Best Practice & Research Clinical Gastroenterology 2016; 30:55-71.

[21] Aragon G, Graham DB, Borum M, Doman DB. Probiotic therapy for irritable bowel syndrome. Gastroenterology & Hepatology 2010; 6:39-44.

[22] McFarland LV. Meta-analysis of probiotics for the prevention of traveler's diarrhea. Travel Medicine and Infectious Disease 2007; 5:97-105.

[23] Guandalini S. Probiotics for prevention and treatment of diarrhea. Journal of Clinical Gastroenterology 2011; 45:S149-53.

[24] Wolvers D, Antoine JM, Myllyluoma E, Schrezenmeir J, Szajewska H, Rijkers GT. Guidance for substantiating the evidence for beneficial effects of probiotics: prevention and management of infections by probiotics. Journal of Nutrition 2010; 140:698S-712S.

[25] Szajewska H, Skórka A. *Saccharomyces boulardii* for treating acute gastroenteritis in children: updated meta-analysis of randomized controlled trials. Alimentary Pharmacology & Therapeutics 2009; 30:960-1.

[26] Szajewska H, Guarino A, Hojsak I, Indrio F, Kolacek S, Shamir R, Vandenplas Y, Wizman Z; European Society for Pediatric Gastroenterology, Hepatology, and Nutrition. Use of probiotics for management of acute gastroenteritis: a position paper by the ESPGHAN Working Group for Probiotics and Prebiotics. Journal of Pediatric Gastroenterology and Nutrition 2014; 58:531-9.
[27] Vandenplas Y. Probiotics and prebiotics in infectious gastroenteritis. Best Practice & Research Clinical Gastroenterology 2016; 30:49-53.
[28] Szajewska H, Skórka A, Ruszczynski M, Gierussczak-Bialek D. Meta-analysis: *Lactobacillus* GG for treating acute gastroenteritis in children – updated analysis of randomised controlled trials. Alimentary Pharmacology and Therapeutics 2013; 38:467-76.
[29] Fang SB, Lee HC, Hu JJ, Hou SY, Liu HL, Fang HW. Dose-dependent effect of *Lactobacillus rhamnosus* on quantitative reduction of faecal rotavirus shedding in children. Journal of Tropical Pediatrics 2009; 55:297-301.
[30] Huang YF, Liu PY, Chen YY, Nong BR, Huang IF, Hsieh KS, Chen KT. Three-combination probiotics therapy in children with *Salmonella* and rotavirus gastroenteritis. Journal of Clinical Gastroenterology 2014; 48:37-42.
[31] Guarino A, Ashkenazi S, Gendrel D, Lo Vecchio A, Shamir R, Szajewska H. European Society for Pediatric Gastroenterology, Hepatology, and Nutrition/European Society for Pediatric Infectious Diseases evidence-based guidelines for the management of acute gastroenteritis in children in Europe: update 2014. Journal of Pediatric Gastroenterology and Nutrition 2014; 59:132-52.
[32] Dubey AP, Rajeshwari K, Chakravarty A, Famularo G. Use of VSL#3 in the treatment of rotavirus diarrhea in children: preliminary results. Journal of Clinical Gastroenterology 2008; 42:S126-9.
[33] Sazawal S, Hiremath G, Dhingra U, Malik P, Deb S, Balck RE. Efficacy of probiotics in prevention of acute diarrhea: a meta-analysis of masked, randomised, placebo-controlled trials. Lancet Infectious Diseases 2006; 6:374-82.
[34] Szajewska H, Kotowska M, Mrukowicz JZ, Armanska M, Mikolajczyk W. Efficacy of *Lactobacillus* GG in prevention of nosocomial diarrhea in infants. Journal of Pediatrics 2001; 138:361-5.
[35] Urbanska M, Gieruszczak-Bialek D, Szajewska H. Systematic review with meta-analysis: *Lactobacillus reuteri* DSM 17938 for diarrheal diseases in children. Alimentary Pharmacology and Therapeutics 2016; 43:1025-34.
[36] Gutierrez-Castrellon P, Lopez-Velazquez G, Diaz-Garcia L, Jimenez-Gutierrez C, Mancilla-Ramirez J, Estevez-Jimenez J, Parra M. Diarrhea in preschool children and *Lactobacillus reuteri*: a randomized controlled trial. Pediatrics 2014; 133:904-9.
[37] Sur D, Manna B, Niyogi SK, Ramamurthy T, Palit A, Nomoto K, Takahashi T, Shima T, Tsuji H, Kurakawa T, Takeda Y, Nair GB, Bhattacharya SK. Role of probiotic in preventing acute diarrhea in children: a community-based, randomized, double-blind placebo-controlled field trial in an urban slum. Epidemiology and Infection 2011; 139:919-26.
[38] McFarland LV. Systematic review and meta-analysis of *Saccharomyces boulardii* in adult patients. World Journal of Gastroenterology 2010; 16:2202-22.
[39] Hempel S, Newbery SJ, Maher AR, Wang Z, Miles JNV, Shanman R, Johnsen B, Shekelle PG. Probiotics for the prevention and treatment of antibiotic-associated diarrhea: a systematic review and meta-analysis. JAMA 2012; 307:1959-69.
[40] Goldenberg JZ, Lytvyn L, Steurich J, Parkin P, Mahant S, Johnston BC. Probiotics for the prevention of pediatric antibiotic-associated diarrhea. Cochrane Database of Systematic Reviews 2015; 22:CD004827.
[41] Szajewska H, Kolodziej M. Systematic review with meta-analysis: *Saccharomyces boulardii* in the prevention of antibiotic-associated diarrhea. Alimentary Pharmacology and Therapeutics 2015; 42:793-801.
[42] Szajewska H, Kolodziej M. Systematic review with meta-analysis: *Lactobacillus rhamnosus* GG in the prevention of antibiotic-associated diarrhea in children and adults. Alimentary Pharmacology and Therapeutics 2015; 42:1149-57.
[43] Szajewska H, Canani RB, Guarino A, Hojsak I, Indrio F, Kolacek S, Orel R, Shamir R, Vandenplas Y, van Goudoever JB, Weizman Z, ESPGHAN Working Group for ProbioticsPrebiotics. Probiotics for the prevention of antibiotic-associated diarrhea in children. Journal of Pediatric Gastroenterology and Nutrition 2016; 62:495-506.
[44] Cimperman L, Bayless G, Best K, Diligente A, Mordarski B, Oster M, Smith M, Vatakis F, Wiese D, Steiber A, Katz J. A randomized, double-blind, placebo-controlled pilot study of *Lactobacillus reuteri* ATCC 55730 for the prevention of antibiotic-associated diarrhea in hospitalized adults. Journal of Clinical Gastroenterology 2011; 45:785-9.
[45] Kolodziej M, Szajewska H. *Lactobacillus reuteri* DSM 17938 in the prevention of antibiotic-associated diarrhea in children: protocol of a randomized controlled trial. BMJ Open 2017; 7:e013928.
[46] Ouwehand AC, DongLian C, Weijian X, Stewart M, Ni J, Stewart T, Miller LE. Probiotics reduce symptoms of antibiotic use in a hospital setting: a randomized dose response study. Vaccine 2014; 32:458:63.
[47] Hickson M. Probiotics in the prevention of antibiotic-associated diarrhea and *Clostridium difficile* infection. Therapeutic Advances in Gastroenterology 2011; 4:185-97.
[48] Goldenberg JZ, Ma SS, Saxton JD, Martzen MR, Vandvik PO, Thorlund K, Guyatt GH, Johnston BC. Probiotics for the prevention of *Clostridium difficile*-associated diarrhea in adults and children. Cochrane Database of Systematic Reviews 2013; CD006095.
[49] Lau CS, Chamberlain RS. Probiotics are effective at preventing *Clostridium difficile*-associated diarrhea: a systematic review and meta-analysis. International Journal of General Medicine 2016; 9:27-37.
[50] Ollech JE, Shen NT, Crawford CV, Ringel Y. Use of probiotics in prevention and treatment of patients with *Clostridium difficile* infection. Best Practice & Research Clinical Gastroenterology 2016; 30:111-8.
[51] Lahtinen SJ, Forssten S, Aakko J, Granlund L, Rautonen N, Salminen S, Viitanen M, Ouwehand AC. Probiotic cheese containing *Lactobacillus rhamnosus* HN001 and *Lactobacillus acidophilus* NCFM modifies subpopulations of fecal lactobacilli and *Clostridium difficile* in the elderly. Age 2012; 34:133-43.
[52] Gao XW, Mubasher M, Fang CY, Reifer C, Miller LE. Dose-response efficacy of a proprietary probiotic formula of *Lactobacillus acidophilus* CL1285 and *Lactobacillus casei* LBC80R for antibiotic-associated diarrhea and *Clostridium difficile*-associated diarrhea prophylaxis in adult patients. American Journal of Gastroenterology 2010; 105:1636-41.
[53] Johnson S, Maziade PJ, McFarland LV, Trick W, Donskey C, Currie B, Low DE, Goldstein EJ. Is primary prevention of *Clostridium difficile* infection possible with specific probiotics? International Journal of Infectious Diseases 2012; 16:e786-92.

[54] Auclair J, Frappier M, Millette M. *Lactobacillus acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 (Bio-K+): characterization, manufacture, mechanisms of action and quality control of a specific probiotic combination for primary prevention of *Clostridium difficile* infections. Clinical Infectious Diseases 2015; 60:S135-44.
[55] Evans CT, Johnson S. Prevention of *Clostridium difficile* infection with probiotics. Clinical Infectious Diseases 2015; 60:S122-8.
[56] Zhang S, Guo J, Liu L. Treating bacteria with bacteria: the role of probiotics in the eradication of *Helicobacter pylori*. International Journal of Clinical Experimental Medicine 2017; 10:4330-41.
[57] Patel A, Shah N, Prajapati JB. Clinical application of probiotics in the treatment of *Helicobacter pylori* infection – a brief review. Journal of Microbiology, Immunology and Infection 2014; 47:429-37.
[58] Dang Y, Reinhardt JD, Zhou X, Zhang G. The effect of probiotics supplementation on *Helicobacter pylori* eradication rates and side effects during eradication therapy: a meta-analysis. PLoS One 2014; 9:e111030.
[59] Lau CSM, Ward A, Chamberlain RS. Probiotics improve the efficacy of standard triple therapy in the eradication of *Helicobacter pylori*: a meta-analysis. Infection and Drug Resistance 2016; 9:275-89.
[60] McFarland LV, Huang Y, Wang L, Malfertheiner P. Systematic review and meta-analysis: multi-strain probiotics as adjunct therapy for *Helicobacter pylori* eradication and prevention of adverse events. United European Gastroenterology Journal 2016; 4:546-61.
[61] Ghouri YA, Richards DM, Rahimi EF, Krill JT, Jelinek KA, DuPont AW. Systematic review of randomized controlled trials of probiotics, prebiotics, and synbiotics in inflammatory bowel disease. Clinical and Experimental Gastroenterology 2014; 7:473-87.
[62] Turner D, Levine A, Escher JC, Griffiths AM, Russel RK, Dignass A, Dias JA, Bronsky J, Braegger CP, Cucchiara S, de Ridder L, Fagerberg UL, Hussey S, Hugot JP, Kolacek S, Kolho KL, Lionetti P, Paerregaard A, Potapov A, Rintala R, Serban DE, Staiano A, Sweeny B, Veerman G, Veres G, Wilson DC, Ruemmele FM, European Crohn's and Colitis Organization, European Society for Pediatric Gastroenterology, Hepatology, and Nutrition. Management of pediatric ulcerative colitis: joint ECCO and ESPGHAN evidence-based consensus guidelines. Journal of Pediatric Gastroenterology and Nutrition 2012; 55:340-61.
[63] Lichtenstein L, Avni-Biron I, Ben-Bassat O. Probiotics and prebiotics in Crohn's disease therapies. Best Practice & Research Clinical Gastroenterology 2016; 30:81-8.
[64] Fedorak RN, Feagan BG, Hotte N, Leddin D, Dieleman LA, Petrunia DM, Enns R, Bitton A, Chiba N, Paré P, Rostom A, Marshall J, Depew W, Bernstein CN, Panaccione R, Aumais G, Steinhart AH, Cockeram A, Bailey RJ, Gionchetti P, Wong C, Madsen K. The probiotic VSL#3 has anti-inflammatory effects and could reduce endoscopic recurrence after surgery for Crohn's disease. Clinical Gastroenterology and Hepatology 2015; 13:928-35.
[65] Singh S, Stroud AM, Holubar SD, Sandborn WJ, Pardi DS. Treatment and prevention of pouchitis after ileal pouch-anal anastomosis for chronic ulcerative colitis. Cochrane Database of Systematic Reviews 2015; CD001176.
[66] Hod K, Ringel Y. Probiotics in functional bowel disorders. Best Practice & Research Clinical Gastroenterology 2016; 30:89-97.
[67] Moayyedi P, Ford AC, Talley NJ, Cremonini F, Foxx-Orenstein AE, Brandt LJ, Quigley EM. The efficacy of probiotics in the treatment of irritable bowel syndrome: a systematic review. Gut 2010; 59:325-32.
[68] Ford AC, Quigley EM, Lacy BE, Lembo AJ, Saito YA, Schiller LR, Soffer EE, Spiegel BM, Moayyedi P. Efficacy of prebiotics, probiotics, and synbiotics in irritable bowel syndrome and chronic iodiopathic constipation: systematic review and meta-analysis. American Journal of Gastroenterology 2014; 109:1547-61.
[69] Guandalini S, Magazzù G, Chiaro A, La Balestra V, Di Nardo G, Gopalan S, Sibal A, Romano C, Canani RB, Lionetti P, Setty M. VSL#3 improves symptoms in children with irritable bowel syndrome: a multicentre, randomized, placebo-controlled, double-blind, crossover study. Journal of Pediatric Gastroenterology and Nutrition 2010; 51:24-30.
[70] Ducrotté P, Sawant P, Jayanthi V. Clinical trial: *Lactobacillus plantarum* 299v (DSM 9843) improves symptoms of irritable bowel syndrome. World Journal of Gastroenterology 2012; 18:4012-8.
[71] Whorwell PJ, Altringer L, Morel J, Bond Y, Charbonneau D, O'Mahoney L, Kiely B, Shanahan F, Quigley EM. Efficacy of an encapsulated probiotic *Bifidobacterium infantis* 35624 in women with irritable bowel syndrome. American Journal of Gastroenterology 2006; 101:1581-90.
[72] Whelan K, Myers CE. Safety of probiotics in patients receiving nutritional support: a systematic review of case reports, randomized controlled trials, and nonrandomized trials. American Journal of Clinical Nutrition 2010; 91:687-703.
[73] Didari T, Solki S, Mozaffari S, Nikfar S, Abdollahi M. A systematic review of the safety of probiotics. Expert Opinion on Drug Safety 2014; 13:227-39.
[74] Salminen MK, Rautelin H, Tynkkynen S, Poussa T, Saxelin M, Valtonen V, Järvinen A. *Lactobacillus* bacteremia, clinical significance, and patient outcome, with special focus on probiotic *L. rhamnosus* GG. Clinical Infectious Diseases 2004; 38:62-9.

Prospects of antimicrobial food packaging in developing countries: processing and food security perspectives

Nichrous Mlalila[1,2,*], **Askwar Hilonga**[3] and **Hulda Swai**[4]

[1] Department of Food Biotechnology and Nutrition Sciences, School of Life Sciences and Bioengineering, Nelson Mandela African Institution of Science and Technology, P.O. Box 447, Arusha, Tanzania
[2] Ministry of Agriculture, Livestock and Fisheries, P.O. Box 9152, Dar es Salaam, Tanzania
[3] Department of Materials Science and Engineering, School of Materials, Energy, Waters and Environmental Science, Nelson Mandela African Institution of Science and Technology, P.O. Box 447, Arusha, Tanzania
[4] Department of Global Health and Biomedical Sciences, School of Life Sciences and Bioengineering, Nelson Mandela African Institution of Science and Technology, P.O. Box 447, Arusha, Tanzania
* Corresponding author: email: nichogm_2006@yahoo.com; mlalilan@nm-aist.ac.tz

Sub-Saharan Africa (SSA) loss about 76% kilocalories (Kcal) of food produced annually before processing, which calls for urgent interventions in order to meet sustainable food security and human development in the region. The interest of international and regional institutions is aligned to increasing the agricultural productivity than reduction of massive food loss in supply chain (FSC), which leads to less sectoral transformations. This paper recommends on development of the fit-for-purpose antimicrobial food packaging materials as the factual agricultural transformation mechanism to stimulate agro-processing and sector diversification in developing countries. It endows with emphasizes on the prospects existing for the industry in SSA from food production volume, rapid urbanization, increase in middle-class society, increasingly young workforces, population growth and post-harvest loss in the region. It is well-known that the global food production is enough to feed the existing population, however due to high food loss in supply system 870 million people is undernourished. Lack of processing and packaging account for 50% of total food lost globally. Critical analysis shows that "without packaging, no processing" and establishment of food processing industries in close proximity to agricultural production settings improves food security and business value chains. Cost-effective and affordable packaging materials show to be the toolkit for nurturing and strengthening local agro-processing industry, improving food security, increasing product competitiveness and influence the production-to-consumption phenomenon.

Keywords: Agro-processing; Fit-for-purpose packaging; Food loss; Food supply chain; Post-harvest loss; Production-to-consumption

1. Introduction

Food security issues arise as special component in 2030 Agenda for Sustainable Development (SDG 2), and human development and ability standards [1]. As food security is manifested through food availability, food accessibility, food utilization and food stability in a particular community, these pillars can be dictated by engaging advanced technologies throughout food supply chain (FSC) [2]. The lack of processing and packaging materials contributes over 50% agricultural produce, which in turn increases incidence of hunger, starvation, malnourishment and diseases. The efforts played by international organizations and developing partners, especially in sub-Saharan Africa (SSA), has led to expanding cultivated land and not productivity [3,4]. The contribution of agricultural sector in food security and poverty reduction, which employs 70-80% of rural population in SSA have changed insignificantly. The cause is very clear that agricultural activities in SSA have realized poor technological transformation in the FSC, which attracts high post-harvest losses (PHL) and poor diversification on the sector. To date, no holistic measures for value addition of food products in changing market economy and lifestyles have been pursued [5]. Technology absorptions have been vulnerable to low economies of scale, socioeconomic issues and the design of many regional and international projects that attached to food productivity for self-sufficient through extension programs and nothing beyond business [6].

In fact, economic growth and development are vital in reduction of poverty and hunger in developing countries. However, scientifically the economic growth and development may have insignificant contribution in intervention of the existing hunger, without traditional-base excellent scientific initiatives and policies [7-9]. Changing paradigm in food processing is iteratively process since 1700s from food salting, sun drying, chilling, fermentation, canning, irradiation, Ohmic heating to emerging nonthermal processing technologies [10]. The current increase in population growth, population resettlement and migration, urbanization, shortage of water and climate change, all need massive contributions of science and technology. As well food security issues should deploy science and technology to give

sustainable food security and cost-effective packaging materials to food producers in SSA. Thanks to current development of active antimicrobials packaging and the application of nanotechnology in food industry, which have promising future to develop packaging materials using locally cheaper and readily available materials in packaging industry [11-13].

In addition, developing sustainable packaging materials could address the contemporary trends in need of mildly processed, preserved food products and guaranteeing the safety and quality of food products [14]. From this perspective, SSA should call for policies to invest in human capital and research facilities and support the absorption of emerging technologies, technology transfer and adoptions for the betterment of the agricultural sector. Investment of science and research within the FSC with scientific consciously implementation of outcomes is important to halt food insecurity in SSA [10]. Development of agricultural sector in Asia is characterized by science and technology ideas supported with national, regional and international policies to create the connectivity of agricultural products and society needs [6,15,16].

The global food production base shows the produced food can feed 7 billion people [10,17]. However, 30-40% of food produced is lost in FSC and most of the food loss occurs in developing countries, especially in SSA [18,19]. It is estimated that 1.3 billion tons of foods, 670 million tons from developed countries and 630 million tons from developing countries [20], are lost and can feed 870 million people each year [9,21,22]. The current food loss and waste need to shift the investment strategies to perceive throughout the FSC, from agriculture for household's self-sufficient agendas to agriculture as business. Increasing the innovative technologies throughout the FSC is a necessary revitalization strategy. Situation analysis shows that investment in food processing and packaging materials would be the core modernization strategies of agricultural sector in SSA as proved in Asian countries [2,12,16,23]. Cost-effective packaging materials is an efficient and effective tool stimulating food processing, reduction of food loss and waste [15,21,23-25]. Packaging at local food producers increases the distribution succession from production-to-consumption and generate more opportunities throughout the FSC [26]. Food service creations in FSC will reduce plague-ridden foods with insects, rodents and contaminating agents like spoilage and pathogenic microorganisms of unprocessed foods [20,27-29]. It worthwhile to divert heavy amount of money channelled to support conventional projects to food processing and packaging for sustainable livelihoods of rural poor people in SSA.

This chapter is restricted to the roles of developing the packaging materials integrated with food preservatives as effective initiative for sustainable food security in developing countries. It entails in the roles of packaging technology and packaging opportunities in SSA through investing in small-scale food producers. In addition, presents the concept of antimicrobial packaging, antimicrobial agents and its relevant global industrial applications. Finally, the paper provides the potential and limitations of new technologies with respect to the scope of this chapter.

2. The roles of packaging technology in food security

Packaging technology in food industry can be defined as the technology materials for containing and protection of food products during their storage, sales, use and distribution [24]. Packaging materials are classified into primary, secondary and tertiary packaging, however, many revolving technologies for extending food shelf-life deal with primary food packaging materials [21,30]. Package comprises of packaging container, food products and headspace. Package protects food products from contact with spoilage microorganisms, chemical contaminants, moisture and oxygen intrusions and other factors such as enzymatic and biochemical reactions [31]. Food packaging, reduce the storage and handling challenges, improve transportation of agricultural products and improves the quality and safety of food products throughout the FSC [16,25]. Therefore, successful food processing industry is dependent to availability of the packaging materials [15]. This ensures that most of produced foods can be available and accessed to consumers and maintains their wellbeing, from food security point of view.

Olsson et al. [30] described packaging as the toolkit for food diversification, which cannot be ignored in FSC and as the engine of food service industry in terms of value addition, functions, customer communication, market differentiation, branding and cost aspects. It is a modernizing agent for outdoor food services including fast food outlets, restaurants, motorway service stations, bistros, cafés, motels and hotels. The food competitiveness depends on the packaging and customer profiling to the packaging materials [30].

3. Food packaging opportunities in developing countries

The global economic contributions of African countries is expected to be 25% by 2050, with the largest global workforce by 2040, increasing middle-class population comprising youth, increase in urbanization and technological advancement.

3.1 Food production and post-harvest losses

According to Manalili et al. [24] investment in packaging industry in developing countries is inevitable. The region contributes 60-80% global food production and has high agricultural growth opportunities than developed world [8]. In addition the qualitative and quantitative PHL in the region is higher accounting as lower as 10-40% and higher as 50-70% [3,19,22,32-35]. In developing countries, the loss is before processing due to lack of storage and handling facilities and transportation while in developed world food loss occurs during retailing, distribution and consumption, hence mainly PHL is determined by technological advancement positioned in FSC [9,10,12,15,20,21,36,37]. **Fig. 2** shows that 39% Kcal of food is lost during production, 37% Kcal during storage and handling, 7% Kcal in processing and packaging, 13% Kcal in distributing and marketing and 7% Kcal during consumption in SSA [29]. Empirically, low food loss during consumption implies low volume of foods enters into such segment. The data is frustrating that 76% of food produced in SSA is lost without reaching the processing and packaging stages. The question remains whether Africa needs agricultural intensifications to meet the sustainable development or not? In which extent the lost food could contribute to poverty alleviation and sustain food security of the community in SSA?

The FSC shows that empirical processing and packaging industry are vital to stabilize the food availability and price volatility in SSA by increasing the availability of foods to the higher segments of FSC from food producers [2,16,25,26,38]. Food and packaging are hotspots for pulling more food products from producers to consumers to address the production-to-consumption phenomena. However, the political structure and socio-economical state transformation should be the key changing factors for the implementation. SSA loss 20-30% of grains which can feed 48 million people per year worth more than US$4 billion annually and exceed the amount of food aid received [22,39]. In Indonesia 6% of rice substantial to feed 204 million of Indonesians are lost every year from rodents [8]. India produces 263 million tons of food and requires on 225-230 million tons per year but 40% of foods is lost worth US$ 8.3 billion [23]. Equally, the annual production data for 2005-2007 from 16 countries of East and Southern Africa for six cereal crops shows that 46.19 million tons were produced and the loss was estimated US$1594 million [34]. The annual production and value of weight loss **(in bracket**) were 27.01 million tons (920 US$ million) maize, 4.72 million tons (139 US$ million) sorghum, 1.67 million tons (60 US$ million) millet, 5.15 million tons (240 US$ million) rice, 5.25 million tons (187 US$ million) wheat and 1.71 million tons (48 US$ million) barley.

For decades no comprehensible and long-term solution has been implemented for intervention of PHL dynamics in FSC, beside various scientific recommendations [7,22,32,33]. Many recommendations and food production programs have been focusing on increasing agricultural production to meet the population growth [8,40], extension services and capacity building to farmers, hoping for more foods without empirical engagement in addressing the PHL and processing issues [3,17,29].

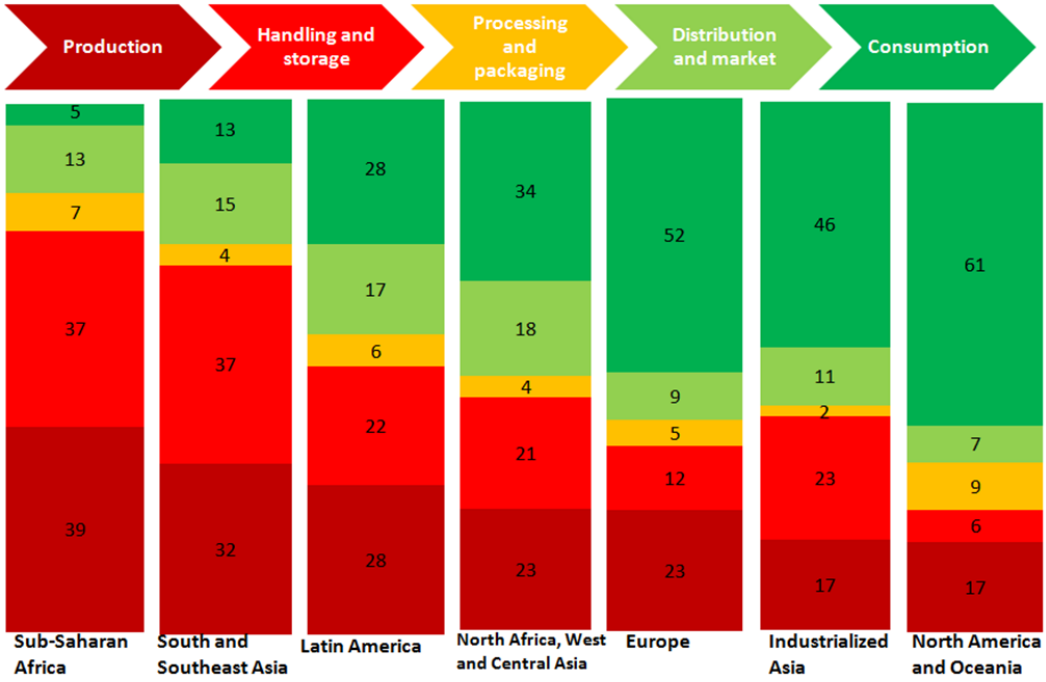


Fig. 1 Global food loss in FSC by region. Modified from [29].

3.2 Population growth and malnutrition

SSA has recorded high population rate of 949 million people and high rural-city movements of people than any region of the world [5,41]. High population and increased of skilled labor in SSA reveal new horizon of investment in agriculture and encourage this society to engage in diverse agricultural production chain. Unpredictability of agricultural activities in rural Africa cause influx of human resettlement and urbanization in Africa pointing other economic production sectors which lead to further adjustment in eating patterns and more food required in cities [16,26]. Poor agricultural diversifications and value chain lead to erratic food prices in both urban and rural areas which cause hunger, starvation, chronic diseases and malnourishment severity [5]. Between 2012 to 2014 Food and Agriculture Organization (FAO) estimated over 12.5% of world population representing 870 million people were chronically undernourished globally and 170 million are children under 5 years of age in SSA [4,10,17,23]. Considering the amount of food lost and the number of people living under severe malnourishment, we can conclude that PHL is the main cause of global malnourishment and food insecurity.

The level of malnourishment in SSA from 1990s to 2014 remains at 218-234 million people [1,36] as the reduction by about 10% from 33.3 to 23.8% compared to same in South-Eastern Asia recorded the reduction of 30.3 to 10.3% [41]. In term of population (in million) the reduction of malnourishment rate from 1990s to 2014 in Africa was 170 to 234, Southern Asia 327 to 304, South-Eastern Asia 134 to 65, Eastern Asia 261 to 167, Latin America and the Caribbean 65 to 49, Western Asia and Northern Africa 13 to 25, Caucasus and Central Asia 9 to 6, and Oceania remained 1 [10]. Stringer, [8] suggested policy-based strategies to combat food insecurity and malnourishment by encouraging international and region to support technological and research investments with increased research infrastructure and human capital. Secondly, technological scale-up of agronomical production constraints in Asia and Africa. And the last is in PHLs control which covers all prospects of food security from food availability to food stability in a particular society. PHL control measures should depict on entire and connect agriculture production to consumption through processing and packaging of agricultural yields [2].

3.3 Food processing and packaging materials

The need of effective packaging materials in development countries is derived from steady increase in food production sector, volume of food produced and increased change in daily diet pressurized high urbanization, population growth and change in daily lifestyle [5,24,26]. Olsson et al. [30] has already mentioned the importance of packaging in food service provisions in maximizing food value, diversification and food safety. In addition, cost-effective and affordable packaging material reflects food processing industry and is the means to ensure smooth

supply of food from production sites to consumers during distribution and marketing [15,21,23,24]. Lack of processing and packaging account for 50% of global food loss in FSC [25,37], which falls in the scope of this chapter.

Manalili et al. [24] showed that, the volume of trade among the developed and developing countries in food packaging materials is equally shared, which in absolute term is off-shore production in vegetable, fruits and other plantation crops [1,24]. Asian countries with successful story of agricultural sector like Indonesia, the Philippines, Thailand, Malaysia and Singapore have heavily invested in food processing and flexible packaging materials to local processors since agricultural green revolution [12,16,23]. Availability of cost-effective packaging materials plays a key role in obsolete modernization of self-sufficient subsistence agricultural model to agricultural business [6,22,32]. The need for processing and packaging of food products aligned with business strategies would be of important in 21st century.

As recommended by United States, [4] governments in SSA, development partners, donors, technical agencies, civil society organizations and private sector supports are needed to create the society with high incomes, well-nourished and increased resilience [2,6]. Equally, dependency on expensive imported packaging materials remains the challenge of progressively agro-processing in SSA. **Fig. 3** shows some packaged foods in Arusha, Tanzania, depicting the levels of packaging materials at various industrial levels. In some cases, homemade and local food producers salvage the water and beverage packaging materials for packaging of juices, honeys and cooking oils among others. This group needs cost-effective fit-for-purpose packaging materials with new functionalities and efficiency.

Fig. 2 Poor and unsafe packaging materials from local agro-processors, which hinder agro-processing competitiveness as taken from Arusha, Tanzania.

Whatever so, the packaging materials used in food packaging demonstrate low competitive edge in marketplace and limit their capability for improved quality standards of the agro-products, which influence consumer discriminatory behavior. It is clear that poor packaging materials are not competitive to the current market and food consumption patterns due to quality loss from microbial deteriorations and environmental stresses. This leads to low contribution of agricultural sector in SSA poverty alleviation strategies and economic development. Therefore, nurturing and strengthening of local agro-processing and packaging industry would improve the agricultural sectors and the need for centralized food production systems with long distribution distances. New processing technologies

and/or upgrading traditional processing practices which align with investments and returns are required to improve food security and malnourishment indices in SSA [7,9,24]. However, the availability of packaging materials would better reflected by building smooth supply connections from production to consumer through market networking, energy stability and affordable transport networks to support the existing and new market efficiently [3,15,17,26].

Manalili et al. [24] proposed to procure the second-hand machinery for producing the friendly packaging materials for ready-to-eat foods with some relaxations on food safety issues. This would increase the production efficiency and produce more acceptable product while staging to the next-production level and building the business chain.

4. Antimicrobial packaging and food preservation strategies

Antimicrobial packaging refers to integration of antimicrobial agents into packaging system for the purpose of preventing microbial growth on food product and extending its shelf life [13,42]. Antimicrobial packaging system aims at (i) food safety guarantee (ii) food quality continuation and (iii) shelf life extension of food products. Beneficial features of antimicrobial packages include extension of shelf life of foods, production of less expensive packages, to reduce the processing labor and use low amount of antimicrobial or produce a free-preservative package. In addition, antimicrobial packaging tends to use low amount of substance to offer maximum protection of food products because less or no inactivation of active substance occur and has localized functional effects to food surface where microorganisms tend to grow [14,43]. The activity of antimicrobials in the packaging causes the extension of the lag phase of target microorganism by contact or by slow release from packaging materials. Longer period release of antimicrobial has been the interest of many research to offer for maintained shelf life, as well as improved quality and safety of food products in FSC [42,44].

The use of antimicrobials in food packaging systems have been motivated with the safety and stability challenges of conventional food packaging. It can be broadly categorized into two main systems namely migratory and non-migratory packaging systems [45,46], however, many of industrial products in the market and active research is on the former system [42,47]. In this respect, developing antimicrobial packaging can solve many challenges of SSA food processing where local agro-processors tend to overdose the foods with preservatives. The practice, make uncompetitive food products and discriminatory consumption system at different social levels. Over-dosage of food preservative makes even more consumer vulnerable to food safety issues with foodborne outbreaks due to microbial gene resistance to preservatives, cancer, gastrointestinal tract disorders and introduction of new pathogens.

Many products show compositional inconsistencies to comply with scientific recommendations due to lack of basic knowledge and skills in food processing such as the use of chemical preservatives. The roles of regulatory authorities on providing technical and scientific recommendations on food safety issue to local entrepreneurs through extension and publicity is definitely low. With introduction of antimicrobial packaging, many packaging limitations such as availability of preservatives and packaging materials will be detached. As in many reports on antimicrobial packaging, other formants of packaging can be used out of integrated antimicrobial onto packaging materials. The use of inserts of sachets/pads in the package into the head space, polymer formulated homogenously or coated with antimicrobial compounds, and immobilization of antimicrobials to the surface of polymers would be useful for to ensure the stability of antimicrobials due to absence of cold chain facilities in many SSA [44,48].

4.1 Food antimicrobial agents

Various antimicrobial compounds are used in developing antimicrobial packaging for food preservation and extending the shelf-life. Antimicrobial compounds have the capability of inhibiting or preventing most of food deteriorating microorganisms. In practice, no broad-spectrum antimicrobial agents against all species of microorganisms like all yeasts, molds, or spoilage microorganisms. They can be used synergetically in order to achieve efficient antimicrobial properties [45]. Both synthetic or naturally extracted compounds are used and have been identified by their broad terms such as organic acids and their salts, bacteriocins, enzymes, macromolecules (chitosan), natural extracts, essential oils and fungicides have been used in synthetic polymers and edible films [48]. Others antimicrobial agents have been from nanomaterials of metals and their salts like titanium, magnesium, copper, silver, zinc, canadium, gold and carbon nanotubes (CNs) [13,31,49].

Applications of bacteriocins, enzymes and essential oils have gained interests due to their broad activity against food spoilage and pathogenic microorganisms [50-53]. Though they have processing stability limitations, their release kinetics from polymers are sustainably controlled. The instability of bacteriocins and enzymes processing conditions and interactions with the food and polymer matrices has led to limited applications in food industry. However, many efforts have been done to setup the processing condition such as pH, temperatures and speciation of

packaging materials. In addition, the uses of essential oils in food packaging have been associated with strong flavor considerations since at optimal activity; tend to suppress the organoleptic threshold levels of food products. The use of metallic nanoparticles and their salts and oxides [46,54-56] as antimicrobial agents in food packaging applications is also promising due to their high stability under processing conditions. However, their applications are limited due to safety queries of the nanoparticles.

During the development of these packaging materials the regulatory concerns and stability suitability in SSA environment should be considered. However, the following general considerations should be taken in account:

(i) The effectiveness of the antimicrobial package(s) against microorganisms in designated type of foods. The best choice of packaging should be that could slow down the growth of microorganisms;

(ii) Effects of the antimicrobial additives on the final physico-mechanical properties on the package or structure. However, the effects of antimicrobial agents depend on the its amount added onto the packages as small amount added is mainly retained in the porous spaces of amorphous polymer;

(iii) Chemical nature of materials and their processing conditions on engineering processing of the package;

(iv) Ultimatum of the action, if leads to reduction of growth rate or cell death of microorganisms;

(v) The diffusion rate of antimicrobial agent(s) from package onto food matrix linking to their toxicological reaction and regulatory concerns; and

(vi) Optimal conditions for antimicrobial activities related to food composition such as pH and acidity.

4.2 Industrial landmarks of antimicrobial packaging

Antimicrobial packaging system for controlling microbial growth on some fields is widely used now in some countries [31,42,47]. In SSA, the USA based-technological company is collaborating with some Africa based companies in manufacturing biocidal mosquito nets in the region. For instance, A to Z Textile Mills Ltd in Tanzania, produces the long lasting mosquito nets to control the malarial endemic in the region. The technology is very visible and the malaria cases have been declining in some regions where the local community complies with use, as are available at subsided costs. The equivalence case can be used for developing new packaging materials incorporated with antimicrobials for food packaging to combat the PHL in developing countries. These packaging should be available at lower cost than market values or at subsidized costs to local entrepreneurs. The impact of developing local agro-processors using affordable technology to them would reduce the PHL, which in turn would improve food security.

Practically, many companies have been involved in manufacturing food related packaging materials incorporated with antimicrobials. Most products have nanomaterial-related ingredients to enhance the antimicrobial activity and performance of the packaging. Common polymers used to incorporate antimicrobials include polyolefin such as LDPE and PP, substituted olefins such as PS, polyesters like PET and polyamides (nylon). Recent literature shows that silver-based antimicrobials and migratory packaging is predominant in the industry ranges from household to consumer products [42,47]. Leading countries in manufacturing of antimicrobial food packaging are Japan and USA and other includes South Korea, China, Brazil, South Africa, and Switzerland (**Table 1**). Despite wide application of antimicrobial packaging in the developed countries, there are much stretched regulations in European countries due to safety uncertainty of antimicrobials to consumers [13,42,46].

Table 1 Summary of some industrial antimicrobial food related packaging products [31,42,45,57].

Trade Name	Antimicrobial	Manufacturing Company
Apacider	Silver zeolite	Sangi (Japan)
Zeomic	Silver zeolite	Shinanen Company (Japan)
Bactekiller	Silver zeolite	Kanebo (Japan)
Silvi Film	Silver oxide	Nimiko Co. (Japan)
Okamoto Super Wrap	Silver oxide	Okamoto Industries, Inc. (Japan)
Ionpure	Silver/glass	Ishizuka Glass Co. (Japan)
WasaOuro	Allyl isothiocyanate	Green Cross Co. (Japan)
Wasa Power	Allyl isothiocyanate	Sekisui Plastic Co. (Japan)
Ageless SE	Silver	Mitsubishi Gas Chem. (Japan)
Acticap	Ethanol	Freund Corporation (Japan)
Take Guard	Bamboo extract	Takex Co. (Japan)
Piatech	Silver oxide	Daikoku Kasei (Japan)
Microban	Triclosan	Microban Products (USA)
BlueMoonGoods™	Silver nanoparticles	BlueMoonGoods, LLC (USA)
MicroGarde	Clove	RhonePoulenc (USA)
MicroFree	Silver, copper oxide, zinc silicate	DuPont (USA)
AgION	Silver	AgION Technologies LLC (USA)
Biomaster®	Silver	Addmaster Limited (USA)
Surfacine	Silver halide	Surfacine Development Co. (USA)
Novaron	Silver zirconium phosphate	Milliken Co. (USA.)
FresherLonger	Nanosilver	Sharper Image (USA)
Baby Mug Cup (	Silver	Baby Dream Co., Ltd., (Korea)
Salad Bowl	Silver nanoparticles	Changmin Chemicals, (Korea)
Biocleanact	Antibiotics	Micro Science Tech Co.(Korea)
NS-315 Water Bottle	Silver	A-DO Global (Korea)
Cleanaid	Silver zeolite	Gyunghyang Ind. Co. (Korea)
Grape Guard	Sulfur dioxide	Quimica Osku S.A. (Chile)
Uvasy	Sulfur dioxide	Grapetek (South Africa)
Ultra-Fresh	Triclosan	Thomson Research Associates (Canada)
Sanitized, Actigard, Saniprot	Triclosan	Sanitized AG/Clariant (Switzerland)

5. Technological potentials and limitations

5.1 Potential of packaging

Many advantageous features of antimicrobial packaging for SSA have been highlighted above. The cost-effective fit-for-purpose antimicrobial packaging material in subsided values embodies two trade-off facets. The commercial and business features represent the first component of packaging materials to community. Small segment of people in rural and outskirts will engage in supply of materials and processing of agro-products which can connect the production segment to consumers out of production settings. This holds diversification aspects of agricultural sector. The second facet is that subsidized cost based on purchasing power will create more specialized agricultural segments and will address the resilience aspects of rural community, especially women and young people to control and modernize FSC from production, processing and packaging to distribution and marketing. This emphasizes the need for early technological adoptions to the large population of people hampered with low economies of scale in SSA [3,9,22].

5.2 Limitations of technological adoptions

The adoption of new technology can be received in mixed sense, that it is always dependent to political will, key stakeholder involvements, socio-economic conditions, cost involved, cultural implications and training and sensitization programs [3]. Political and key stakeholders' involvements are the key to success in any program to influence the adoption and technical backstopping of the program and market networking [9,41]. Compressive analysis shows that, the agendas on global reduction of food loss and waste are nearly triggered with strikes in food prices. Starting from 1975 to 1985 PHL reduction received high attention due to price volatility of grains in 1974 and the first World Food Conference redefined food security concept and the United Nations General Assembly adopted in its Seventh Special Session in 1975 to halt by 50% of food loss and waste by 1985 [9,20,41]. At that

time, many SSA established Food Reserve Bureaus to oversee the availability of foods in their countries especially for grain [9,19,41]. After food price stabilization in 1985 many supporting nationals including Japan, UK, Canada, Denmark, Germany, Australia, USA, FAO, UNDP pulled out their support. The food crisis in 2007/08 caused riots in some 23 countries (14 in SSA) which revived again the agenda of reduction in food loss and waste [4,6,10,32]. Some efforts have been placed rigorous restructuring the existing food reserve bureaus and formation of Unclassified Food Reserve institutions to provide the early warning on the state of foods. With currents erratic droughts and El-Nino rainfall in SSA could intensify the scenarios of food insecurity redressing through mitigation of food loss [7].

Therefore, the PHLs of food might be not well addressed globally through sustainable strategies at both regional and international levels. If the issue of PHL could be addressed continuously from 1970s to 2000s, the reduction of food loss and waste could be well addressed and more foods would be available to consumers. This need joint efforts from producers, food producers, governments, regional and international institutions and private sector to reach the well-health and food secured society.

6. Conclusion: future trends in packaging

The amount of food loss within FSC globally is the current challenge to attain the sustainable food security and poverty alleviation in developing countries and especially in SSA. Also, the global population living under severe malnourishment because of low availability of food is large. However, food produced in the world is enough to sustain the current population. Government leaders, regional and international institutions should come up with tangible solutions of food loss. Conventional policies of increasing agricultural productivities have stressing the environment and ecosystem due to increased putting land under cultivation and application of chemicals. Beyond that food processing and packaging have benchmark in assisting in agricultural sector development as during the green revolution in Asian countries. Putting more efforts in processing and packaging of food products engage more people in FSC, stabilize food products, make food safer to consumers and make small farmers to think beyond self-sufficient food production scenarios. This means, without deliberate efforts in investing throughout FSC using scientific proved strategies in PHLs control, the world will not advance the food security issues and feed the hungry population.

Present understanding on the stages in which high food loss occur can help to develop more rigorous strategies and determine the efforts required to be integrated to address the problem and other related factors in the agricultural sector. In SSA where food processing and packaging marks at the lowest record, with unsafe foods, severe malnourishment, hunger, chronic diseases and food insecurity; cost-effective fit-for-purpose packaging materials are needed. Antimicrobial packaging integrated with preservatives is advantageous to address various food processing and packaging bottlenecks in SSA. The packages reduces the extra-cost of buying preservatives, can be easily used by unskilled labors and extend the shelf-life of food products for longer time compared to conventional food preservation methods. This increases margin values of food, increases food competitiveness edge, provides safer food, healthier and more nutritional food products. Therefore, antimicrobial packaging can be the revolutionization toolkit of FSC during transport, storage, processing, packaging, and distribution and finally as motivating agent for agro-processing industry in SSA.

Opportunities to establish packaging industry in SSA are many. It records, high population growth, high urbanization and high proportion of arable land for food production. With availability of packaging material agricultural production would connect more farmers that are specialized, processors, distributors and business. The production-to-consumption scenario in local agricultural production will lead to competitive food service industry for internal and international trade. Equally, it reduces the food lost unprocessed, reduces hunger and starvation and ultimate makes sustainable livelihood, health and food security. At large, the kind of packages is of paramount in this era of global food insecurity, high PHLs, global population increase, climate change, and increased competition of water for agriculture and domestic use.

Acknowledgment The support by the Commission for Sciences and Technology (COSTECH), Government of Tanzania and NM-AIST is gratefully acknowledged.

References

[1] Conceição, P.; Levine, S.; Lipton, M.; Warren-Rodríguez, A. Toward a food secure future: Ensuring food security for sustainable human development in Sub-Saharan Africa. *Food Policy*. **2016**; 60 1-9

[2] Augustin, M. A.; Riley, M.; Stockmann, R.; Bennett, L.; Kahl, A.; Lockett, T.; Osmond, M.; Sanguansri, P.; Stonehouse, W.; Zajac, I.; Cobiac, L. Role of food processing in food and nutrition security. *Trends in Food Science & Technology*. **2016**; 56 115-125

[3] Stewart, K.; Smith, R.; Abruzzese, L.; Steiner, H.; Shallcross, W.; Ouvry, P.; Kenny, M. Global food security index 2016: An annual measure of the state of global food security. The Economist Intelligence Unit: *The Economist Group*. http://foodsecurityindex.eiu.com/Index; Accessed on: 13/09/2016.

[4] United States. A Food-Secure 2030: A Global Vision and Call to Action. *United States Agency for International Development (USAID)*. Washington, DC. **2016**; https://feedthefuture.gov/sites/default/files/resource/files/A_Food-Secure_2030_0.pdf.

[5] Kimenju, S. C. and Qaim, M. The nutrition transition and indicators of child malnutrition. *Food Security*. **2016**; 8 (3): 571-583

[6] Pelletier, B.; Hickey, G. M.; Bothi, K. L.; Mude, A. Linking rural livelihood resilience and food security: an international challenge. *Food Security*. **2016**; 8 (3): 469-476

[7] John, B.-A. More scientific thinking needed to feed society: The NSTF tackles hunger. *South African Journal of Science* **2016**;

[8] Stringer, R. Food Security Global Overview. In M. Caraher and J. Coveney (Eds.), Food Poverty and Insecurity: International Food Inequalities. Cham: *Springer International Publishing*. 2016. http://dx.doi.org/10.1007/978-3-319-23859-3_2.

[9] Shafiee-Jood, M. and Cai, X. Reducing Food Loss and Waste to Enhance Food Security and Environmental Sustainability. *Environmental Science & Technology*. **2016**; 50 (16): 8432-8443

[10] Buckle, K. Can Food Science Reduce World Hunger? In S. Hongladarom (Ed.), Food Security and Food Safety for the Twenty-first Century: Proceedings of APSAFE2013. Singapore: *Springer Singapore*. 2015. http://dx.doi.org/10.1007/978-981-287-417-7_1.

[11] Imran, M.; Revol-Junelles, A.-M.; Martyn, A.; Tehrany, E. A.; Jacquot, M.; Linder, M.; Desobry, S. Active Food Packaging Evolution: Transformation from Micro- to Nanotechnology. *Critical Reviews in Food Science and Nutrition*. **2010**; 50 (9): 799-821

[12] Kagan, C. R. At the Nexus of Food Security and Safety: Opportunities for Nanoscience and Nanotechnology. *ACS Nano*. **2016**; 10 (3): 2985-2986

[13] Mlalila, N.; Kadam, D. M.; Swai, H.; Hilonga, A. Transformation of food packaging from passive to innovative via nanotechnology: concepts and critiques. *Journal of Food Science and Technology*. **2016**; 53 (9): 3395–3407

[14] Azlin-Hasim, S.; Cruz-Romero, M. C.; Morris, M. A.; Cummins, E.; Kerry, J. P. Effects of a combination of antimicrobial silver low density polyethylene nanocomposite films and modified atmosphere packaging on the shelf life of chicken breast fillets. *Food Packaging and Shelf Life*. **2015**; 4 26-35

[15] Marsh, K. S. Packaging for Enhanced Food Security. Food Safety Magazine. February/March, 2012. http://www.foodsafetymagazine.com/magazine-archive1/februarymarch-2012/packaging-for-enhanced-food-security/; Accessed on: 12/09/2016.

[16] Jeremiah, D. and Low, C. *Growing Processed Food Industry and Rising Demand for Food Exports Boost the Southeast Asian Flexible Plastic Packaging Market*. Singapore: Frost & Sullivan. 2009. http://www.frost.com/prod/servlet/press-release.pag?docid=168776387; Accessed on: 12/09/2016.

[17] McKenzie, F. C. and Williams, J. Sustainable food production: constraints, challenges and choices by 2050. *Food Security*. **2015**; 7 (2): 221-233

[18] Godfray, H. C. J.; Beddington, J. R.; Crute, I. R.; Haddad, L.; Lawrence, D.; Muir, J. F.; Pretty, J.; Robinson, S.; Thomas, S. M.; Toulmin, C. Food security: the challenge of feeding 9 billion people. *Science*. **2010**; 327 (5967): 812-818

[19] Parfitt, J.; Barthel, M.; Macnaughton, S. Food waste within food supply chains: quantification and potential for change to 2050. *Philosophical Transactions of the Royal Society B: Biological Sciences*. **2010**; 365 (1554): 3065-3081

[20] Prusky, D. Reduction of the incidence of postharvest quality losses, and future prospects. *Food Security*. **2011**; 3 (4): 463-474

[21] Verghese, K.; Lewis, H.; Lockrey, S.; Williams, H. Packaging's Role in Minimizing Food Loss and Waste Across the Supply Chain. *Packaging Technology and Science*. **2015**; 28 (7): 603-620

[22] Affognon, H.; Mutungi, C.; Sanginga, P.; Borgemeister, C. Unpacking Postharvest Losses in Sub-Saharan Africa: A Meta-Analysis. *World Development*. **2015**; 66 49-68

[23] Babu, V. Enhancing food security through sustainable packaging.Mumbai: *Business Standard*. February 11, 2016. http://www.business-standard.com/content/b2b-plastics-polymers/enhancing-food-security-through-sustainable-packaging-116021100146_1.html; Accessed on: 12/09/2016.

[24] Manalili, N. M.; Dorado, M. A.; Otterdijk, R. Appropriate food packaging solutions for developing countries: *Food and Agricultural Organization (FAO)*: Rome, Italy. 2014: pp. 37

[25] Opara, U. L. and Mditshwa, A. A review on the role of packaging in securing food system: Adding value to food products and reducing losses and waste. *African Journal of Agricultural Research*. **2013**; 8 (22): 2621-2630
[26] Wenban-Smith, H.; Faße, A.; Grote, U. Food security in Tanzania: the challenge of rapid urbanisation. *Food Security*. **2016**;
[27] Ji, S.; Lu, J.; Liu, Z.; Srivastava, D.; Song, A.; Liu, Y.; Lee, I. Dynamic encapsulation of hydrophilic nisin in hydrophobic poly (lactic acid) particles with controlled morphology by a single emulsion process. *Journal of Colloid and Interface Science*. **2014**; 423 85-93
[28] Blanco-Padilla, A.; Soto, K. M.; Hernández Iturriaga, M.; Mendoza, S. Food Antimicrobials Nanocarriers. *The Scientific World Journal*. **2014**;
[29] Lipinski, B.; Hanson, C.; Lomax, J.; Kitinoja, L.; Watie, R.; Searchinger, T. Reducing food loss and waste: Working Paper, Installment 2 of "Creating a Sustainable Food Future". *World Resources Institute*: Washington, DC. 2013: pp. 40
[30] Olsson, A.; Petterson, M.; Jönson, G. Packaging demands in the food service industry. *Food Service Technology*. **2004**; 4 (3): 97-105
[31] Echegoyen, Y. Nano-developments for Food Packaging and Labeling Applications. In M. Rai, C. Ribeiro, L. Mattoso, and N. Duran (Eds.), Nanotechnologies in Food and Agriculture. Switzerland: *Springer International Publishing*. 2015.
[32] Kaminski, J. and Christiaensen, L. Post-harvest loss in sub-Saharan Africa—what do farmers say? *Global Food Security*. **2014**; 3 (3–4): 149-158
[33] World Bank. Missing food: the case of postharvest grain losses in sub-Saharan Africa. *World Bank*. Washington DC. **2011**; 116
[34] Hodges, R. J.; Buzby, J. C.; Bennett, B. Postharvest losses and waste in developed and less developed countries: opportunities to improve resource use. *The Journal of Agricultural Science*. **2011**; 149 (S1): 37-45
[35] Abass, A. B.; Ndunguru, G.; Mamiro, P.; Alenkhe, B.; Mlingi, N.; Bekunda, M. Post-harvest food losses in a maize-based farming system of semi-arid savannah area of Tanzania. *Journal of Stored Products Research*. **2014**; 57 49-57
[36] Bahadur, K. C., Krishna ; Haque, I.; Legwegoh, A.; Fraser, E. Strategies to Reduce Food Loss in the Global South. *Sustainability*. **2016**; 8 (7): 595
[37] Olsmats, C. and Wallteg, B. Packaging is the answer to world hunger. World Packaging Organisation (WPO) and International Packaging Press Organisation (IPPO): *WPO/IPPO*. http://www.worldpackaging.org/i4a/pages/index.cfm; Accessed on: 12/09/2016.
[38] Floros, J. D.; Newsome, R.; Fisher, W.; Barbosa-Cánovas, G. V.; Chen, H.; Dunne, C. P.; German, J. B.; Hall, R. L.; Heldman, D. R.; Karwe, M. V.; Knabel, S. J.; Labuza, T. P.; Lund, D. B.; Newell-McGloughlin, M.; Robinson, J. L.; Sebranek, J. G.; Shewfelt, R. L.; Tracy, W. F.; Weaver, C. M.; Ziegler, G. R. Feeding the World Today and Tomorrow: The Importance of Food Science and Technology. *Comprehensive Reviews in Food Science and Food Safety*. **2010**; 9 (5): 572-599
[39] Tefera, T. Post-harvest losses in African maize in the face of increasing food shortage. *Food Security*. **2012**; 4 (2): 267-277
[40] Beddington, J. Food security: contributions from science to a new and greener revolution. *Philosophical Transactions of the Royal Society B: Biological Sciences*. **2010**; 365 (1537): 61-71
[41] Mvumi, B. M. and Stathers, T. E. *Food security challenges in Sub-Saharan Africa: The potential contribution of postharvest skills, science and technology in closing the gap*, in *11th International Working Conference on Stored Product Protection* 2015, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand: Bangkok. p. 32-43.
[42] Bumbudsanpharoke, N. and Ko, S. Nano-Food Packaging: An Overview of Market, Migration Research, and Safety Regulations. *Journal of Food Science*. **2015**; 80 (5): R910-R923
[43] Mellinas, C.; Valdés, A.; Ramos, M.; Burgos, N.; Garrigós, M. d. C.; Jiménez, A. Active edible films: Current state and future trends. 2016; http://dx.doi.org/10.1002/app.42631.
[44] Radusin, T.; Skrinjar, M. M.; Cabarkapa, I. S.; Pilic, B. M.; Novakovic, A. R.; Hromis, N. M. Actual and future trends in antimicrobial food packaging. *AgroFood Industry Hi-Tech: Food Safety*. **2013**; 24 (4): 44-49
[45] Corrales, M.; Fernández, A.; Han, J. H. Antimicrobial Packaging Systems. In J. H. Han (Ed.), Innovations in Food Packaging (Second Edition). San Diego: *Academic Press*. 2014. http://dx.doi.org/10.1016/B978-0-12-394601-0.00007-2.
[46] Kuorwel, K. K.; Cran, M. J.; Orbell, J. D.; Buddhadasa, S.; Bigger, S. W. Review of Mechanical Properties, Migration, and Potential Applications in Active Food Packaging Systems Containing Nanoclays and Nanosilver. *Comprehensive Reviews in Food Science and Food Safety*. **2015**; 14 (4): 411-430
[47] Vance, M. E.; Kuiken, T.; Vejerano, E. P.; McGinnis, S. P.; Hochella, M. F.; Rejeski, D.; Hull, M. S. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. *Beilstein journal of nanotechnology*. **2015**; 6 (1): 1769-1780
[48] Rudra, J. S.; Dave, K.; Haynie, D. T. Antimicrobial polypeptide multilayer nanocoatings. *Journal of Biomaterials Science, Polymer Edition*. **2006**; 17 (11): 1301-1315
[49] Díez-Pascual, A. M. and Díez-Vicente, A. L. Poly (3-hydroxybutyrate)/ZnO bionanocomposites with improved mechanical, barrier and antibacterial properties. *International journal of molecular sciences*. **2014**; 15 (6): 10950-10973
[50] Valdes, A.; Mellinas, A. C.; Ramos, M.; Burgos, N.; Jimenez, A.; Garrigos, M. C. Use of herbs, spices and their bioactive compounds in active food packaging. *RSC Advances*. **2015**; 5 (50): 40324-40335
[51] Shariatinia, Z. and Fazli, M. Mechanical properties and antibacterial activities of novel nanobiocomposite films of chitosan and starch. *Food Hydrocolloids*. **2015**; 46 112-124

[52] Asbahani, A. E.; Miladi, K.; Badri, W.; Sala, M.; Addi, E. H. A.; Casabianca, H.; Mousadik, A. E.; Hartmann, D.; Jilale, A.; Renaud, F. N. R.; Elaissari, A. Essential oils: From extraction to encapsulation. *International Journal of Pharmaceutics*. **2015**; 483 (1-2): 220-243

[53] Morelli, C. L.; Mahrous, M.; Belgacem, M. N.; Branciforti, M. C.; Bretas, R. E. S.; Bras, J. Natural copaiba oil as antibacterial agent for bio-based active packaging. *Industrial Crops and Products*. **2015**; 70 134-141

[54] Cushen, M.; Kerry, J.; Morris, M.; Cruz-Romero, M.; Cummins, E. Silver migration from nanosilver and a commercially available zeolite filler polyethylene composites to food simulants. *Food Additives & Contaminants: Part A*. **2014**; 31 (6): 1132-1140

[55] Azlin-Hasim, S.; Cruz-Romero, M. C.; Ghoshal, T.; Morris, M. A.; Cummins, E.; Kerry, J. P. Application of silver nanodots for potential use in antimicrobial packaging applications. *Innovative Food Science & Emerging Technologies*. **2015**; 27 136-143

[56] Díez-Pascual, A. M. and Díez-Vicente, A. L. ZnO-Reinforced Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) Bionanocomposites with Antimicrobial Function for Food Packaging. *ACS Applied Materials & Interfaces*. **2014**; 6 (12): 9822-9834

[57] Ayhan, Z. Potential Application of Nanomaterials in Food Packaging and Interactions of Nanomaterials with Food. In C. Silvestre and S. Cimmino (Eds.), Ecosustainable Polymer Nanomaterials for Food Packaging: Innovative Solutions, Characterization Needs, Safety and Environmental Issues. Boca Raton, FL: *Taylor & Francis Group, LLC*. 2013.

Shelf life of banana, orange and mango in polystyrene containers within refrigerated chamber

L. Partida Ruvalcaba[1,*], **V. Eng Felix**[1], **F. Ayala Tafoya**[2], **T. de J. Velazquez Alcaraz**[2], **T. Diaz Valdes**[2], **M.G. Yañez Juarez**[2] and **R. Medina Lopez**[2]

[1] Career in Sustainable and Protected Agriculture, Technological University of Culiacan, km 2 Culiacan-Imala road, col. Los Angeles, PC 80014, Educating, Sustainable and Knowledge City, Culiacan, Sinaloa, Mexico

[2] Faculty of Agronomy, Autonomous University of Sinaloa, km 17.5 Culiacan-Eldorado road, PO Box 25, PC 80000, Culiacan, Sinaloa, Mexico. *Corresponding author: parpolo@yahoo.com.mx.

The objective of this research was to determine the shelf life of bananas, oranges and mangoes stored in sealed polystyrene containers within a refrigerated chamber with temperature 3-9 °C for oranges and 10-12 °C for bananas and mangoes, high relative humidity (85%) and renovation frequent of the air by the chamber compressor. Oranges were harvested directly from the orchard and washed with water with 150 mg L^{-1} sodium hypochlorite. The bananas were collected at the Culiacan market in Sinaloa, Mexico, with maturity indexes 1, 2, 3 and 4, and they were then washed with chlorinated water at 5 mg L^{-1} and dried manually. Mangoes were acquired when they were in the index of physiological maturity. When bananas with maturity index 1 were stored in polystyrene containers closed, their shelf life was 21 days until showed the maturity index four, but control who were confined in the bottom tray of the refrigerated chamber were badly damaged. Oranges quality was preserved and shelf life was extended for more than 100 days without symptoms of deterioration. Mangoes had shelf life of 70 days to submit the sensory maturity index, while controls were badly damaged.

Keywords: Postharvest; Quality; Color; Firmness; Soluble Solids

1. Introduction

One of the most popular fruits in Mexico is orange, of which one of the most cultivated varieties is 'Valencia', which goes into production in May, is juicy, sweet and its production is oriented to the production of juice. During the last ten years, 341 thousand hectares were allocated for this crop, with an average annual growth of 0.2 percent. In 2007 the orange harvest was just over four million tons, which placed the country in the fourth place in the world, surpassed only by Brazil, the United States of America and India. Veracruz is the leading state in orange production in Mexico, followed by Tamaulipas, San Luis Potosí, Puebla and Nuevo León, although in Sonora it is where the highest national yield has been recorded, with 25 t ha^{-1}, which has allowed exporting to the United States of America, Japan, the United Kingdom and other European countries [1]. Banana (*Musa paradisiaca*) is a fruit of general consumption in Mexico due to its nutritional value, availability all year round and its relatively low price. The average production from 2000 to 2010 was 2'111,800 t and the respective total and per capita consumption were 2'037,909 t and 19.7 kg. [2], while mango (*Mangifera indica* L.) in 2006 produced 1.7 million tons in 172,000 hectares, and the main producing states were Sinaloa, Guerrero, Nayarit, Oaxaca, Chiapas, Veracruz and Michoacán, which contributed 90% of the production [3].

There is an increasing consumer demand for high quality fruits and vegetables. Locally–sourced produce that visually appeals to the buyer at the time of purchase and is still attractive and tasty upon consumption is considered to be high quality. The ideal solution is to preserve the overall quality (organoleptic, commercial, microbiological and nutritional) of horticultural products and to meet the demands of the market by to improving post-harvest storage life [4][5][6]. Improving post-harvest handling and therefore, the quality and shelf life of oranges, the Mexican producers will be better able to take advantage of market windows and increased profitability in its production system.

Post-harvest storage techniques, employed once the fruits have been packed for fresh marketing, are intended to preserve the fruit quality by maintaining ideal environmental conditions. These conditions reduce respiration and promote a longer shelf-life which allows for shipments to distant markets with reduced loss. Among the techniques used for preserving fruits and vegetables are cooling, the use of controlled atmospheres, ethylene absorbers, application of opaque films, and exogenous application of plant growth regulators [7].

Respiration is the main physiological process that leads to the deterioration of the fruit. Respiration is attenuated by low temperatures that reduce the respiratory rate, reduce excessive loss of water, and slow biochemical and enzymatic reactions. Maturity is a physiological and biochemical process that is under genetic and hormonal controls, is a process that is accompanied by multiple changes at the cellular level, rather than an increase in size [8]. The respiration rate of the fruit is reduced by half for every 10 °C reduction in temperature [9]. In climacteric fruits such as mango, temperatures above 40 °C cause an increase in respiratory activity; by contrast, temperatures below 13 °C decrease respiration and prolong shelf life [10]. Respiration-induced water losses in fruits cause a reduction of fruit weight, leading to a decrease in the fruit quality and acceptability [11], these losses often lead to higher losses than 5% during marketing, up to 7% in cold storage for three months and commercialization [11]. The low humidity conditions lead to

increased transpiration and therefore a high loss of water, which accelerates fruit senescence and a marked loss of quality, both wrinkles in the cortex as shrinkage and softening [9].

During postharvest storage, fruit senescence can lead to a loss of fruit quality, including flesh softening, loss of acidity, reduction of vitamin C and changes in organoleptic characteristics (taste and palatability) [12]. The reaction rate of metabolic processes, which lead to loss of quality, doubles for every 10 °C increase in temperature, and in the range of 0 to 10 °C may even six-fold [12].

There are limitations as to the minimum temperatures that can be applied in cold storage. Some tropical and subtropical fruit show sensitivity to low temperatures, which is manifested by different alterations and spots on the skin, usually known as injury or chilling injury and can cause significant loss of merchantable quality [12]. Fruits should not be frozen while in storage. Fruits and vegetables for fresh consumption must maintain an active metabolism, which can only be achieved in the liquid phase, so they cannot be subjected to temperatures below freezing between 0 and -1.5 °C [12].

Fruits in cold storage should be stored under high humidity to avoid dehydration [9]. The appropriate relative humidity for a given product depends on the surface to volume ratio. As this ratio increases, respiration increases as well. A relative humidity level between 85 and 95% is advisable to achieve the goal of conservation [9]. Postharvest Giant Cavendish bananas (AAA) have been shown to increase shelf life when stored it's in polystyrene containers closed within a refrigerated chamber at temperatures of 10-12 °C [13].

The objective of this research was to determine the efficacy of polystyrene containers sealed to increase the shelf life bananas, oranges and mangoes stored inside a refrigerated chamber with temperature 3-9 °C for oranges and 10-12 °C for bananas and mangoes, high relative humidity (85%) and renovation frequent of the air by the chamber compressor.

2. Bananas

After that the bananas with maturity index three were confined in closed polystyrene containers inside the refrigerated chamber with a temperature of 10-12 and 8-10 °C inside the containers, they expressed the characteristic yellow color of the maturity index four, up to five days after the packaging, until that date (March 10, 2015) showed typical symptoms of organoleptic or sensorial maturity, consisting of brown spots on the epicarp of the bananas. The above indicates that biochemical reactions such as respiration were diminished by the controlled atmosphere (little oxygen and CO_2, cold environment and no air circulation) in the polystyrene containers; That is to say, in the conditions of the mentioned atmosphere the concentration of oxygen was very little and, consequently, the generations of CO_2 and ethylene also were very limited, since in conditions closed, cold and to exist little oxygen, as mentioned Salisbury and Ross [14], metabolic reactions of methionine may also have been very rare to generate ethylene, a hormone that induces maturation of climacteric fruits such as banana. Furthermore, according to Martinez [12], in cold fruits it tends to decrease respiration and, therefore, the consumption of O_2 and the production of CO_2.

When the bananas (control) were stored in the lower tray of the refrigerated chamber (Figure 1), at the end of the same period (March 6-10, 2015) it was observed that the fruits showed very pronounced signs of organoleptic or sensory maturity (brown spots on the pericarp); however, through the touch and the manual pressure it was perceived that the firmness of the pulp of the fruits was similar to that of those that were packaged in the containers of polystyrene. The flavor was another of the characteristics similar to that of the fruits packaged in the mentioned containers. However, the appearance of bananas with brown spots on a large part of the pericarp, would surely inhibit their acquisition or purchase by the consumer.

Figure 1 shows how the bananas with the maturity index two were stored in the polystyrene containers, with a temperature of 10-12 °C inside the refrigerated chamber and 8-10 ˚C inside the containers, had a life of shelf of 12 days until March 31, date in which they presented the index of maturity four. This also confirms that in bananas confined to this type of containers (controlled atmosphere) placed inside the cooler with cold environment, breathing decreased and, as a consequence, also decreased the production of ethylene. However, in the same figure it can be observed that when the bananas were confined in the lower tray of the refrigerated chamber with a temperature of 10-12 °C, by March 31, 2015, they had brown-colored pericarp, although with consistency and flavor acceptable for consumption.

When the bananas had the maturity index three and on April 16 were stored in the polystyrene containers inside the refrigerated chamber with the temperatures mentioned above, they took nine days to present the maturity index four, without in the epicarp appeared the symptoms of sensory maturity. According to Wills *et al*. [8], it can be deduced that of the two systems responsible for the synthesis of ethylene, system 1 initiated the synthesis of ethylene but failed to trigger the operation of system 2 to allow the synthesis of large quantities of ethylene necessary for full integration of maturation processes and acceleration of the maturity of bananas.

Contrary to what was observed in the bananas placed in the closed polystyrene containers and the same temperature inside the refrigerated chamber (10-12 °C), the fruits that were deposited in the lower tray of the same chamber showed the symptoms of sensory maturity (brown spots on the epicarp) six days after storage, indicating that, according to Wills *et al*. [8], system 1 triggered the synthesis of ethylene in greater quantities which made the bananas have a faster maturation process compared to the one in the closed polystyrene containers.

Within the closed polystyrene containers with a temperature of 10-12 °C inside, the bananas with four maturity index were stored and after 10 days they showed the symptoms of sensory maturity or brown spots. Contrary to what happened with the bananas inside the polystyrene containers, in the control that were deposited in the lower tray of the refrigerated chamber, to the ten days the epicarp was of dark color which made that the bananas no longer would look good for marketing. Fruits with maturity index one, which were also stored inside the containers with 8-10 °C of temperature inside, and 21 days later reached the index of maturity four, becoming well known that the ethylene production system 1 did not trigger the generation of this hormone in the large amounts typical of system 2, which according to Wills *et al*. [8] delayed the ripening of the fruits.

Color, firmness and taste deteriorate as time passes, and as the Brix grades increase from 26.1, 39.6 and 87.4%, reaching the indexes two, three and four, respectively, in comparison with index one; but while reaching the index two bananas surpassed with 26.1% in Brix degrees to the average of the index one (green), those that reached index three surpassed in those of the index two and those that reached index four surpassed in 34.2% to those of index three. However, when the bananas began to show brown spots on the cover, the Brix degrees decreased and the fruits already presented ethanolic condition or with some fermentation.

The results in the change of color, firmness, flavor and degrees Brix had to be product of phenomena like the action of the ethylene, the perspiration and the breathing, that although they were diminished by the low temperatures and the null circulation of air inside the containers of polystyrene, did not cease to appear during the life of the bananas.

a b c d E

Fig. 1 Bananas with maturity index two that were stored on March 20, 2015 in polyestyrene containers (a), same fruits on day 24 (b), 30 (c), 31 (d) and control on day 30 (e).

Table 1 shows how the color, firmness and taste deteriorate as time passes, and as the Brix degrees increased from 26.1, 39.6 and 87.4%, reaching indexes two, three and four, respectively, in comparison with index one; but while reaching the index two the bananas outperformed in 26.1% degrees Brix to the average of the index one (green), those that reached index three exceeded in 10.7% to those of the index two and those that reached the index four exceeded in 34.2% to those of index three. However, when the bananas began to show brown color spots on the cover, Brix degrees decreased and the fruits already presented ethanolic condition or with some fermentation. The results in the change of color, firmness, flavor and degrees Brix had to be product of phenomena as the action of the ethylene, the perspiration and the respiration, that although they were diminished by the low temperatures and the null circulation of air inside of the polystyrene containers closed, they did not stop presenting during the life of the bananas.

Table 1 Sensorial characteristics in four maturity indexes of the banana 'Giant Cavendish' (AAA).

Maturity index	Color	Firmness	Flavor	°Brix
Index 1	G	Strong	Tasteless	11.1 e*
Index 2	GY	Strong	Little sweet	14.0 d
Index 3	YPG	Strong	Sweet	15.5 b
Index 4	YT	Half	Strong sweet	20.8 a
Brown spots on cuticle	YB	Weak	Ethanolic	14.4 c
DMSH	----	----	----	0.24

*Means with different letter in the same column are statistically different (Tukey, α=0.05). G=green, GY=yellow green, YPG=yellow with green peduncle, YT=total yellow, YB=yellow with brown spots. DMSH=Honest Significant Minimal Difference.

3. Orange

The skin color of freshly harvested oranges (0 days) was light yellow, but after 90 and 100 days of storage the color was bright yellow or bright orange (Figure 3). After 90 and 100 days of refrigerated storage in polystyrene containers, the peel luminosity increased. After 90 and 100 days of refrigerated storage in polystyrene containers, there was a significant increase in the peel luminosity. Peel luminosity increased by 4.2 and 4.4 respectively, compared to the freshly harvested fruit (Figure 2). This corresponded to a 6.1 and 6.4% increase. Similarly, there was significant

increase in peel luminosity of the stored fruit compared with the refrigerated controls, 6.1 and 6.4% respectively, compared to the freshly harvested fruit (Table 2), and it increased by 4.6 and 4.8% respectively, compared with the refrigerated control. The hue angle, which defines the color, decreased by 0.8% in oranges stored for 90 days, and 9.3% in those with 100 days of stored and 2.0% in the freshly harvested oranges, compared with the refrigerated controls (Table 2), but the trend was toward the yellow (<90). The chromaticity, indicating the color purity, is increased by 2.0 and 5.0%, respectively, in oranges stored at 90 and 100 days, compared to the chromaticity of the controls, but for oranges freshly harvested increases the increase was 7.3 and 10.4%, respectively. The hue angle, which defines the color, only had a statistically significant difference in oranges stored for 100 days in polystyrene containers, decreased 7.3 in those with 100 days of storage and 0.6 in oranges stored for 90 days and 1.6 in oranges freshly harvested, compared with the refrigerated controls (Table 1), but the trend was towards yellow (<90). This corresponds to a decrease of 0.8% Hue angle oranges stored for 90 days, 9.3% those with 100 days of storage and 2.0% in the freshly harvested oranges.

Fig. 2 Orange cv. Valencia harvested on 04/02/2015 in Culiacan, Sinaloa, Mexico.

A b C

Fig. 3 Condition of orange cv. Valencia stored for 90 (a) and 100 (b) days in sealed polystyrene containers under refrigeration, or stored and refrigerated for 100 days (c) in the tray of refrigerated chamber.

Table 2 Components of color (L, °Hue and C) in orange peel 'Valencia' freshly harvested, stored for 90 and 100 days in polystyrene containers or 100 days in the bottom tray of the refrigerated chamber (control).

Treatments	Luminosity (L)	Hue angle (°Hue)	Chromaticity (C)
0 days	69.1 b*	77.0 a	70.1 c
90 days	73.3 a	78.0 a	75.2 b
100 days	73.5 a	71.3 b	77.4 a
Control	70.1 b	78.6 a	73.7 b

*Means with the same letter in each column are statistically equal (Tukey, $\alpha \leq 0.05$).

Chromaticity, indicating the color purity is increased by 1.5 and 3.7 respectively in oranges stored at 90 and 100 days, compared to the chromaticity control but over freshly harvested oranges the increase was 5.1 and 7.3, respectively, there was statistically significant difference between the control and stored for 100 days and as well as with the freshly harvested, however no significant difference with stored for 90 days and control refrigerated. It increased 2.0% in 90 days and 5.0% 100 days storage in polystyrene containers, compared to control chromaticity, but with respect to freshly harvested oranges the increase was 7.3% 90 days and 10.4% 100 days of storage. The pulp (endocarp) luminosity increased significantly by 10.0% in the oranges stored for 100 days compared with the refrigerated controls, by 8.8% compared with the freshly harvested fruit (Table 3). However, °Hue decreased 11.2 and 12.5% compared with those obtained in the freshly harvested and in control; however, the color hue (°Hue) oranges stored up to 90 days was not different from that of the freshly harvested and refrigerated control fruit.

Table 3 Components of the color (L, °Hue and C) pulp of oranges 'Valencia' freshly harvested, stored for 90 and 100 days in polystyrene containers or 100 days in the bottom tray of the refrigerated chamber (control).

Treatments	Luminosity (L)	Hue angle (°Hue)	Chromaticity (C)
0 days	45.4 b*	85.4 b	26.7 a
90 days	43.6 c	86.0 ab	21.9 b
100 days	49.4 a	75.8 c	23.2 b
Control	44.9 b	86.6 a	23.3 b

*Means with the same letter in each column are statistically equal (Tukey, $\alpha \leq 0.05$).

The chromaticity of oranges stored up to 90 and 100 days in polystyrene containers also varied with respect to the refrigerated control, although it should be noted that their values were lower by 18.0, 13.1 and 12.7%, respectively, compared with freshly harvested fruit. Considering that the negative or positive value of a* indicates color from green to red and the negative or positive values of b* indicate color from blue to yellow, Table 4 shows that the values of a* were positive but much lower than those of b*, which also confirms the bright yellow in the peel (epicarp) and pulp (endocarp) of oranges.

These results indicate that the increase in the yellow of the oranges stored in polystyrene containers increased sensory perception, since according to Schvab *et al*. [15], there is a relationship between sensory perception of sweet and acid flavors with the intensity of color, yellow and green in oranges. In addition, with the use of closed polystyrene containers at an internal temperature of 6-7 °C in refrigerated chambers operated at temperatures 3-9 °C, peel coloration increased, which coincides with the work of Ariza *et al*. [16], who found that the yellow hue intensifies with increasing low temperatures because of the promotion of carotenoid synthesis that leads to the coloring of the orange. Ariza *et al*. [16], also stated that there was a need for post-harvest technologies that degreening the fruit without the use of chemicals. In our work, it was the use of polystyrene containers and low temperatures that cause the degreening, yet do not contaminate the fruit and, consequently, do not harm the health of consumers or harm the environment.

Table 4 Color parameters (a* and b*) of oranges ('Valencia') freshly harvested, stored for 90 and 100 days in polystyrene containers or refrigerated chamber control.

Temperature	Parameter a* Peel	Parameter b* Peel	Parameter a* Pulp	Parameter b* Pulp
0 days	16.0 b*	68.2 b	2.1 b	26.7 a
90 days	15.6 bc	73.5 a	1.5 c	21.8 b
100 days	25.4 a	73.3 a	5.7 a	22.5 b
Control	14.1 c	66.4 b	1.4 c	23.3 b

*Means with the same letter in each column are statistically equal (Tukey, $\alpha \leq 0.05$).

Results from Table 5 show that the polar diameter of the oranges were affected by the process of random selection, however, also it shows that the polar diameter of the refrigerated control fruit decreased by 7.9% compared to the fruit stored for 90 days, and by 10.0% compared to the fruit stored for 100 days in containers. The equatorial diameter of the refrigerated control fruit decreased by a respective 9.4 and 8.3% compared with oranges stored for with 90 or 100 days. This indicates that the refrigerated control oranges experienced a significant loss of water through transpiration.

Fruit firmness and Brix are two quality characteristics that will decrease with the passage of time, so for the refrigerated control oranges, there was a significant firmness decrease of 18.1% compared to those fruit stored for 90 days and 8.0% compared to fruit stored for 100 days (Table 5). However, the average of total soluble solids (°Brix) was not affected by the storage. Total soluble solids is one of fruit quality parameters most frequently evaluated in citrus, and is seen as a critical indicator of sensory quality [17]. It has been shown that there is a good correlation between sensory attributes of acid and sweet taste with total acidity, expressed as percentage of citric acid and the concentration of total sugars of the juice [15]. According to our work, the Brix of the oranges stored in polystyrene containers, did not decrease compared to the Brix of refrigerated control fruit, as the Brix values were statistically similar. Oranges stored in polystyrene containers better preserved their appearance compared to oranges were only refrigerated, these differences can be seen easy, with this information available preservation appetizing appearance of oranges stored in polystyrene containers is an opportunity for mexican producers of oranges, which may extend their selling season storing oranges in this system, it can reduce losses by accelerated ripening oranges and sell them for more time to market.

Table 5 Polar and equatorial diameter, firmness and °Brix orange 'Valencia' freshly harvested, stored for 90 and 100 days in polystyrene containers or 100 days in the bottom tray of the refrigerated chamber (control).

Treatments	Polar diameter (cm)	Equatorial diameter (cm)	Firmness (N)	°Brix
0 days	8.2 b*	7.2 b	14.0 ab	15.4 a
90 days	8.8 a	8.5 a	15.5 a	12.8 b
100 days	9.0 a	8.4 a	13.8 b	11.9 b
Control	8.1 b	7.7 b	12.7 b	12.7 b

*Means with the same letter in each column are statistically equal (Tukey, $\alpha \leq 0.05$).

4. Mango

In a first experiment it was observed that the fruits of the control sample were ripe and soft in texture at the end of 13 days after they were confined in the lower tray of the refrigerated chamber, since these fruits were stored on June 12 and revised until the 24th of the same month of 2015.

However, the mangoes stored in polystyrene containers had excellent appearance and good pulp consistency. This indicates that biochemical reactions such as respiration were diminished by the modified atmosphere (low oxygen and CO_2, cold environment and no air circulation) in the polystyrene containers; That is to say, in the conditions of the mentioned atmosphere the concentration of oxygen was very little and, consequently, the generations of CO_2 and ethylene also were very limited, since in conditions closed, cold and to exist little oxygen, as mentioned Salisbury and Ross [14], the metabolic reactions of methionine may also have been very rare to generate ethylene, a hormone that induces maturation of climacteric fruits such as mangoes. In addition, according to Martinez [12], in cold fruits tends to decrease respiration and, therefore, the consumption of O_2 and the production of CO_2.

In a second experiment some fruits (control) were also stored in the lower tray of the refrigerated chamber (06/25/2015), and after 19 days the fruits were ripe with a very soft pulp, as the mangoes were checked until the 13th July, 2015. Unlike the appearance of the control mangoes, those that were stored in polystyrene containers had very good appearance in color and consistency of the pulp. This also confirms that in the mangoes confined in this type of containers (modified atmosphere) placed inside the refrigerated chamber with cold environment, breathing decreased and, therefore, the production of ethylene also decreased.

So according to Wills *et al*. [8], it can be deduced that in the mangoes within the polystyrene containers, system 1 of ethylene synthesis started, but failed to trigger the operation of system 2 to produce the synthesis of large quantities of ethylene necessary for the full integration of maturation processes and acceleration of mangoes maturity.

In a third experiment, the refrigerated chamber it was closed during 70 days, and the fruits within of the polystyrene containers, that were placed in inside of the same chamber, were have excellent shelf life (Figure 4), with good color, consistency, maturity grade and flavor.

Fig. 4 Mangoes cv. Kent, 70 days after they stored in polystyrene containers with temperature of 10-12 °C in refrigerated chamber and 8-10 °C inside of the polystyrene containers collocated inside refrigerated chamber.

In the first experiment the analysis of variance indicated that the weight of the fruits had an average of 427.6 g, standard deviation (S) of 42.7 g, coefficient of variation (CV) equal to 9.9% and a probability (Pr) of committing error of 0.0001. But in Table 1 it can be observed that the weight of the mangoes decreased as the storage days passed, so that the control fruits decreased their weight by 28.6% compared to the fruits that had six days of storage in containers of polystyrene, and 24.7% in relation to the fruits that had 13 days confined in the same containers; however, the weight difference between the fruits reviewed at 6 and 12 days was only 5.2%, which in turn indicates that within the containers the weight loss was very low perhaps due to the scarce air circulation in compared to the air circulation in the bottom tray of the refrigerated chamber.

In the second experiment the mean fruit weight was 394.0 g, S=38.4 g, CV=9.8% and Pr=0.0001; however, the same tendency also occurred in the reduction of fruit weight, and the difference of this was 24.6% of the control fruits compared to those who had six days of storage in the polystyrene containers, while compared with fruits with 13 days

stored in the same containers the difference was 12.3%, and the weight difference between those stored for 6 and 13 days was 14.1%.

In the first experiment, mean fruit length was equal to 11.0 cm, S=0.7 cm, CV=6.4% and Pr=0.0006, but perspiration (loss of water) fruits decreased 12.0% in the control mangoes compared to those stored in the closed polystyrene containers (Table 6), while the width of the mangoes had a mean of 7.9 cm, S=0.22 cm, CV=2.8% and Pr=0.0001, with a decrease of 11.9% in the controls compared to those that were stored in the polystyrene containers.

In the second experiment the length of the mangoes was practically unchanged (Table 6), but the mean of the sample was 14.1 cm, S=0.29, CV=0.28 and Pr=0.0726; however, statistical differences were observed in the width of the fruits, with a mean of 3.1 cm, S=0.17 cm, CV=5.6% and Pr=0.0102, such that this character decreased 9.1% in the control fruits, in compared to those stored in polystyrene containers.

According to the analysis of variance, the Brix degrees (°Brix) had a mean of the sample equal to 17.9, S=0.77, CV=4.3% and Pr=0.0001. However, in this case the °Brix increased in the control mangoes (Table 6), since after six days after their storage in the lower tray of the refrigerated chamber, these fruits had 74.0% more total soluble solids than the fruits that were stored in polystyrene containers.

Table 6 Weight, length, width and °Brix observed in the control mangoes and stored in polystyrene containers.

	First experiment			Second experiment			
Days of storage	Weight (g)	Length (cm)	Width (cm)	Weight (g)	Length (cm)	Width (cm)	°Brix
6	482.0 a*	11.7 a	8.4 a	452.3 a	14.3 a	3.3. a	13.1 b
12	456.8 a	-----	-----	388.7 b	-----	-----	-----
Control	344.1 b	10.3 b	7.4 b	340.9 c	14.2 a	3.0 b	22.8 a
DMSH	56.8	0.7095	0.2241	45.5	0.2823	0.1767	0.7785

*Means with different letter in the same column are statistically different (Tukey, α=0.05).

4. Conclusions

The shelf life of bananas increased at the four maturity indices after they were washed with sodium hypochlorite solution (5.0 mg L^{-1}) and stored in closed polystyrene containers and placed in a cool temperature chamber in such a way that the principle of thermal insulation of the polystyrene is fulfilled, so that it can be added as a further technology in the procedure to increase the shelf life of the banana.

The tone and purity of yellowing of the skin and pulp of oranges increased significantly within polystyrene containers and, consequently, its life was extended to more than 100 days, they had symptoms of impairment loss of polar and equatorial diameter or decrease in total soluble solids, compared to those characteristics of oranges from the bottom tray of the refrigerated chamber. Inside containers closed polystyrene temperature decreased to two degrees, with respect to the temperature inside the refrigerated chamber, so that the principle of thermal insulation polystyrene reasserted, and its usefulness for packaging products such as fruits and solve some of their own physiological problems, to prolong its shelf life and enhance your presentation. This technology could be exploited by Mexican citrus producers which have more shelf life in its oranges to store them in polystyrene containers at low temperatures.

The polystyrene containers were efficient in the post-harvest handling and in the prolongation of the shelf life of the mango fruits, so that this type of packaging can be used as a technology added to the hitherto applied ones (fresh temperature, controlled atmosphere and refrigerated chamber) to preserve mango fruits for longer.

References

[1] Gob.mx [Internet]. Sistema de Informacion Agropecuaria y Pesquera, SAGARPA. [cited 2015 Dec 27]. Available from: http://www.siap.sagarpa.gob.mx/.

[2] García, M. R., González M. M. F., García S. R. C., Mora F. J. S., González E. A. y Martínez D. A. 2013. El mercado del plátano (*Musa paradisiaca*) en México, 1971-2017. Agrociencia 47: 399-410.

[3] SIAP, Servicio de Información Agroalimentaria y Pesquera. 2008. Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA). Producción Agrícola 2006. http://www.siap.gob.mx/agricola_siap/ientidad/index.jsp(23/04/2008).

[4] Artes F. Innovations modulated physical treatments to preserve fruit and vegetable quality post-harvest. II. Cyclic thermal treatments. Science Magazine Food Technology. 1995; 35(2):139-149.

[5] Artes F. Advances in postharvest conservation fresh lemon and grapefruit treatments. Agricultural Levante. Special post-harvest. 1999; 348:289-294.

[6] Artes F. Conservation of plant products in modified atmospheres. *In*: Lamua M, editor. Application of cold food. MundiPrensa; 2000. p. 105-125.

[7] Parikh HR, Nair GM, Modi VV. Some structural changes during ripening of mangoes (*Mangifera indica* var. Alphonso) by abscisic acid treatment. Annals of Botany. 1990; 65:121-127.
[8] Wills R, McGlasson B, Graham D, Yoice D. Introduccion a la fisiologia y manipulacion poscosecha de frutas, hortalizas y plantas ornamentales. 2a Edicion, Zaragoza: Acribia, SA; 1998. p. 36-37.
[9] Guerra F. Tecnologia post-cosecha de frutos citricos. Curso integral de citricultura. Instituto de Investigaciones de Fruticultura Tropical. 1996. p. 242-257.
[10] Ponce de Leon L. Bozquez storage techniques. Post-harvest handling handle. Ed. EMEX. A. C. 1997; p. 22-24.
[11] Jimenez CM, Martinez JJM, Cuquerella J. Plastic individual sear-packaging of spanish fruit. Proceedings of the XV International Congress of Refrigeration, Commission C2. 1983; 460-466.
[12] Martinez JJM. Frigoconservacion in oranges and tangerines. Phytoma Rev. 1997; 90:136-140.
[13] Reyes PDZ, Ruvalcaba LP, Mares VA. Management of the banana Cavendish Giant (AAA) in postharvest for increasing its shelf life. Open Access Library Journal. 2015; 2(12):1-9.
[14] Salisbury FB, Ross CW. Plants physiology. Paraninfo Thomson Learning, Madrid, España. 2000; 988 p.
[15] Schvab MC, Maria MF, Liliana MG, Cristina VD. Quality parameters entrerrianas orange juice. Revista Iberoamericana Postharvest Technology. 2013; 14(1):85-92.
[16] Ariza FR, Tejacal IA, Beltran MN, Ambriz CR, Lugo AA, Barrios F, Barbosa M. Quality of fruits orange 'Valencia' in Morelos, Mexico. Revista Iberoamericana Postharvest Technology. 2010; 11(2):148-153.
[17] Matheis JP, Fellman KJ. Influencing factors preharvest flavor of fresh fruit and vegetable. Postharvest Biol Tech. 1999; 15:227-232.

Study of the migration phenomena from milk polypropylene bottles

Z. Bouchaib[1], **M.O. Boussoum**[1,2], **N. Belhaneche-Bensemra**[2] and **R. Dahak**[2]

[1] Faculte des Sciences de la Nature et de la Vie, Université Ibn Khaldoun, Tiaret, Algeria
[2] Laboratoire des Sciences et Techniques de l'Environnement, Ecole Nationale Polytechnique, BP 182 El-Harrach, Alger, Algeria

The aim of this work is to study the interactions between polypropylene (PP) bottles and milk.. For that purpose, the migration tests were carried out for 10 days at two different temperatures (20 and 40°C), with and without agitation and by using two food simulants: n-octane and sunflower oil. The mass variation of PP samples with time was followed. The phenomena of migration were studied by using Fourier transform infrared spectroscopy in attenuated total reflexion mode (FTIR-ATR), atomic absorption spectrometry (AAS) and gas chromatography- mass spectrometry (GC-MS), .

The results showed the influence of contact time, temperature, storage conditions and simulant nature on migration phenomena from food package. .

Keywords: PP; migration; milk; liquid simulant; FTIR-ATR; AAS

1. Introduction

The presence, in the food or in the environment, low quantities of potentially toxic products is it dangerous to man? In the absence of scientific certainty, can we establish a legislation?

This is the reason for which the units of scientific research are focusing more on the safety and quality of food packaged to protect the health of the consumer. In effect, packaging play a revealing and indicator role of the life level of societies. By its technological and marketing functions, the packaging makes life less requiring it would be essential for the consumer. But, it can cause damaging nuisance that it must nearby look, even if we can't prove the existence of a cause-effect relationship between exposition to a substance and the appearance of a disease for the low doses.

It is in this context that the study of the content containing interactions has extended the field of food to the pharmaceutical, hereafter the scientific evidences made at the end of the 60 years involving the packaging as a source of foods contamination [1].

At front of the multiplicity of products, the constraints of conservation, the amenities of use and today the requirements related to the environment, there is a wide variety of materials and packaging systems forming part of our lives [2]. In effect, a direct contact of a plastic packaging with a food can be a source of reciprocal interactions between content and container causing quality defects; flavor or aroma of food is able to pass from the product to the outside (aromatic loss). In addition, additives contained initially in the polymer can also migrate to the food; then there is contamination of the product [3-7].

Our work is devoted precisely to highlight the context of the migration of additives contained initially in the bottles of milk, to study the factors favoring this process and finally to identify the migrating substances.

In this work, migration testing have been carried out in two food simulants, sunflower oil and n-Octane, with and without agitation a tow different temperature. The migration phenomenon has been firstly analyzed by a preliminary study based on the rate of mass variation of samples which were in contact with the two food simulants at 20 and 40°C and using three analytical techniques of analysis Fourier transform infrared (FTIR-ATR) atomic absorption spectrometry (AAS) and gas chromatography-mass spectrometry (CG-MS).

2. Materials and Methods

2.1 Migration testing

Samples having a diameter of 20 ± 0.1 mm and average mass (0, 1600±0.001) g were cut from the selected PP bottle into squares. Migration tests were carried out for 10 days at two different temperatures (20 and 40°C), with and without agitation and by using two food simulants: n-octane and sunflower oil (directive 82/711EEC). Ten samples of PP were immersed in 100 ml of food simulant. A squares sample and 10 ml of food simulant were taken off every day. The rate of mass variation was calculated according to the following equation:

$$\tau(\%) = [(m_t - m_0)/ m_0] * 100 \qquad (1)$$

Where:

m_0= initial mass before immersion

m_t = mass of the sample at the time t.

The weights were measured to an accuracy of 10^{-4}g.

2.2 FTIR-ATR spectroscopy analysis

This technique has been used for the purpose to confirm the nature of the polymer used in the manufacture of the selected bottles and to perform a semi-quantitative estimation of the migration phenomenon by identifying the migrant species. A polymeric film (PP) was recovered and analyzed directly with a Perkin–Elmer model *Spectrum One*. The resolution was 2 cm^{-1}.

2.3 AAS Atomic absorption spectrometry analysis

PP samples were first mineralized out as follows [8]:

- Weigh a 0.4 g sample in a porcelain crucible.
- Insert the crucible in a muffle furnace at 900 ° C for 2 hours until white ash is obtained.
- Leave the crucibles cool.
- Add 2 mL of supra pure nitric acid.
- Dissolve the residue with distilled water and complete with the same solvent up to 20 mL.

The concentrations of Zn, Pb, Cu, and Cd were determined using a Perkin–Elmer analyst spectrometer.

2.4 GC-MS analysis

GC-MS analysis was performed on a Perkin–Elmer GC connected with a MS detector. A 30 m capillary column PE-5MS (5% diphenyl, 95% dimethyl polysiloxane), i.d = 0.25 mm; df = 0.25 μm, Perkin–Elmer) was used. The analysis was carried out using electron impact mode and an ionization potential of 70 eV. The carrier gas was helium with a flow of 2 ml/min.

The separation of DOA from PP was done by Soxhlet extraction with chloroform according to the method developed by Wang and Storm [9]. The analysis was conducted under the following conditions: 90 °C held for 3min, heated up to 250 °C at a rate of 6 °C/min and held for 13min. Molecular mass in the range 50–450 amu was scanned. The identification of different peaks was deduced by searching in the MS library (NIST) and further confirmed by running the known chemical for DOA

Calibration curve for DOA was prepared in chloroform at concentrations that covered the concentration range found in the polymer extracts. The resulting line was linear with correlation coefficient of 0.9977. Three analytical replicates were analyzed for each concentration.

3. Results and discussion

3.1 Study of changes in the rate of mass change

The rate of mass variation as function of time gives information about the phenomenon which occurred between the samples and the food stimulants.

Figure 01show the evolution of the rate of mass variation with time of contact under the influence of agitation and a temperature in the case of the sun flower oil and n-octane. It can be noted that (τ) increase during the first 6 day of contact who indicate the penetration of the two liquid simulants in the free volume. Then after this duration the shape of all curves decreases, which confirm that migration of additives occurred in both food simulants. However, we note that the rate of mass variation is higher in the case of the sun flower oil. Due to the good solubility of the plasticizer in fatty stimulant, and also to its low solubility in aqueous stimulant [10, 11].

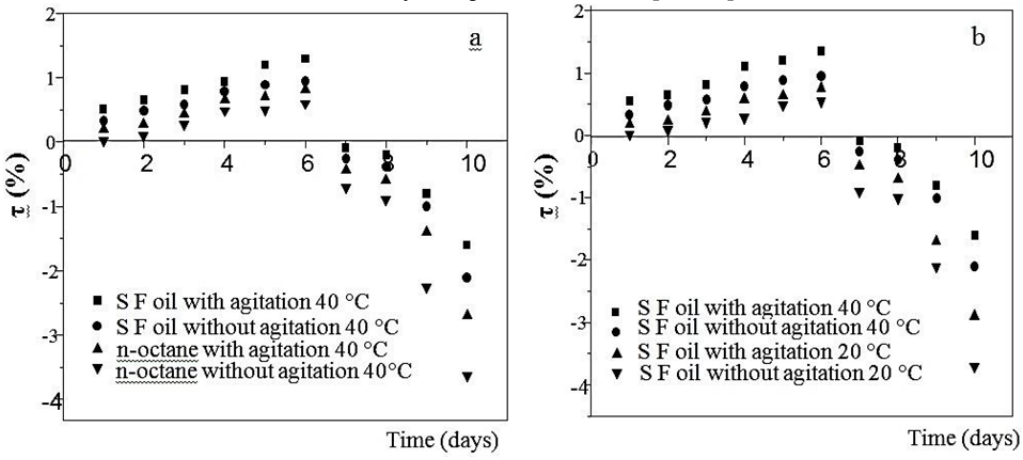


Fig. 1 Evolution of the rate of mass variation versus time of contact in the case of sunflower oil and n-octane with and without agitation at 20 and 40 °C. **a)** highlight the influence of the agitation and the nature of food simulant; **b)** highlight the influence of temperature

The study of the rate of mass variation allows us to deduce:

- The food stimulant has a real influence on the phenomenon of migration.
- The agitation affects the variation of mass in the tow food stimulant by the renewal of the contact surface of the plastic material.
- The temperature is an important factor favoring the phenomena of interaction between polypropylene (PP) bottles and milk.

3.1.1 Estimate of the overall migration

The table 1 shows the values of the overall migration in the sun flower oil and in the n-octane. All values of overall migration determined are lower than the maximum overall migration established by the EEC: 10 mg/dm^2, which means that the migration has not affected the quality of the food stimulant.

Table 1 Values of overall migration in mg/dm$^{2.}$

	Sunflower oil, 40°C		**n-octane, 40°C**		**Sunflower oil, 20°C**	
	With agitation	Without agitation	With agitation	Without agitation	With agitation	Without agitation
Overall migration (mg/dm^2)	-0.434	-2.745	-2.998	-2.810	-1.496	-3.894

In addition, the smaller values of overall migration are obtained in the case of samples having been in contact with the sunflower oil; this can be explained by the good solubility of plastizer in oil while its solubility in the n-octane is very low due to the low viscosity of the latter, which confirmed the influence of the nature of food stimulants in the migration phenomenon. Moreover, the most important rate of migration was observed at 40 °C under the influence of agitation and a temperature. After the results showed, we can conclude that all factors studied have a real influence on the phenomenon of migration.

3.2 Migration analysis by infrared spectroscopy

In order to follow the evolution of the characteristics bands of additives present in the formulation of the polymer constitutive of this bottle we have study the ATR spectra of samples of bottles were in contact with food simulants at 0 day (witness), 6th day and 10th day at two different temperatures 20 and 40°C with and without agitation.

A semi quantitative estimation of the migration of additives was done. For that purpose, the following absorbance's ratios were calculated: A 1166 /1372, A 1338/1372, A 1455/1372 and A 2853/1372

In our case, we considered the band at 1372 cm^{-1} corresponding to the vibration of the deformation of CH_3 of PP [12] as the more stable band which is taken as a reference band.

An initial increase is first observed in all absorbance ratios indicating the penetration of the food simulant in the PP samples, followed by a decrease of all absorbance ratios, their decrease in intensity corresponds to a migration of one or more components in the food simulants. The report calculated in the case of tests with agitation are lower than those measured for the tests without agitation. Most of the monomers and additives are lipophilic, and the migration is a function of the affinity between the migrant and the packaged product, migration is generally more important in a fat medium than in an aquous.

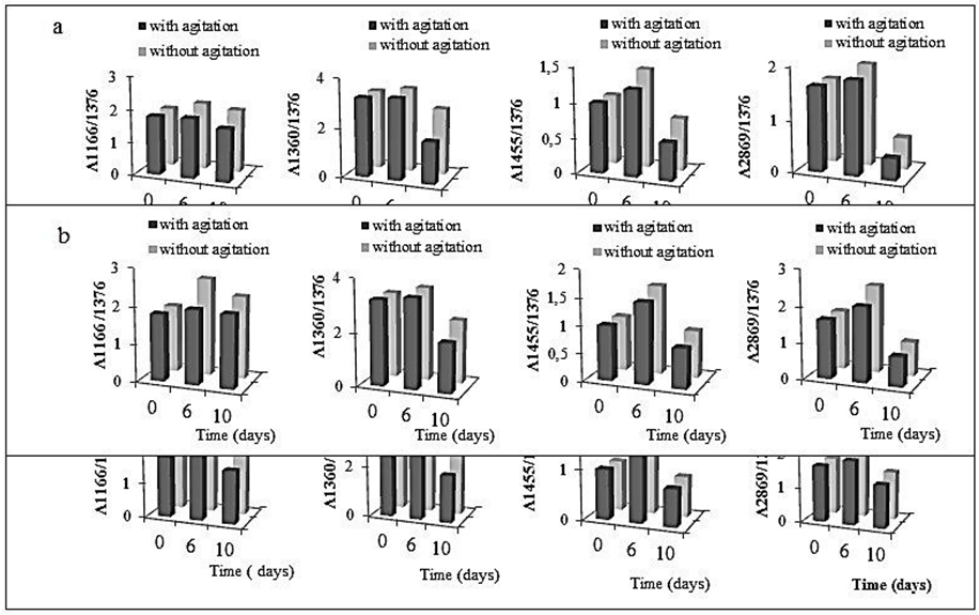


Fig. 2 Variation of absorbance's ratios as a function of time of contact with: a) Sunflower oil at 40°C; b) Sunflower oil at 20°C; c) n-octane at 40°C

3.3 Atomic absorption spectrometry analysis

The atomic absorption spectrometry was first used to determine the concentration of Zn, Pb, Cd and Cu in the PP samples. The results showed in Tables 2, 3, and 4 shows their presence. The presence of this element would be linked to the additives present in the formulation or to residues of the polymerization (catalysts). In addition, the presence of zinc is probably due to the fact that the heat stabilizer used during the manufacture of the bottle is zinc stearates; its characteristics bands have been also detected by infrared spectroscopy.

Table 2-4 Concentrations (µg /l) of metals present in the samples which were in contact with: Sunflower oil (40°C and 20°C) and n-octane at 40°C.

2	**Sunflower oil (40°C)**							
	With agitation				**Without agitation**			
Concentration (µg/L)	[Zn]	[Pb]	[Cu]	[Cd]	[Zn]	[Pb]	[Cu]	[Cd]
2 days	610	0,31	61,5	11,07	1465	1,32	132,7	11.61
4 days	550	0,21	55,5	6,69	1325	1,15	96,5	10.63
6 days	510	0,15	52,6	5,06	780	0,82	77,4	7.35
8 days	450	0,11	48	4,78	635	0,38	65,05	5.68
10 days	380	0,08	37,6	4,69	410	0,10	55,25	5.31

3	**Sunflower oil (20°C)**							
	With agitation				**Without agitation**			
Concentration (µg/L)	[Zn]	[Pb]	[Cu]	[Cd]	[Zn]	[Pb]	[Cu]	[Cd]
2 days	1290	1,91	183,4	10,70	1915	1,78	260,9	14,89
4 days	1110	1,75	122,05	8,35	1805	1,77	200,7	12,795
6 days	1270	1,685	56	6,635	1720	1,76	41,8	12,235
8 days	980	1,535	50,60	6,505	1575	1,74	40	9,515
10 days	685	1,185	45,39	6,455	1490	1,70	37,55	8,77

4	**n-octane (40°C)**							
	With agitation				**Without agitation**			
Concentration (µg/L)	[Zn]	[Pb]	[Cu]	[Cd]	[Zn]	[Pb]	[Cu]	[Cd]
2 days	1600	1.03	226.4	8.33	1975	3.08	448.4	17,17
4 days	1545	0.70	102.3	5.71	1720	1.23	334.4	12,58
6 days	1495	0.50	50.25	5.67	1680	0.65	151.1	12,23
8 days	1325	0.35	47.5	4.85	1405	0.60	141.1	8,50
10 days	1355	0.31	32.9	4.66	1255	0.55	103.5	8,16

According to the results shown by the Tables 2, 3 and 4 It can be noted that the residual concentrations in the PP samples decreased with time. By comparison between the values showed in case of samples which were in contact with sunflower oil and those found in the n-octane, we note that there is an effect of affinity which shows the influence of the food simulant on the migration phenomenon. In addition, the comparison of the results shown in tables 2 and 3 confirm the influence of the temperature and the agitation which increase the mobility of the molecules of the additives and promote their migration in the food simulants.

3.4 Application of the GC/MS to the study of the specific migration

In order to identify the various additives contents in the composition of the formulation of the milk bottles having been used for migration tests, we proceeded to the analysis of the pellet witness and those having undergone migration testing in the two environments simulators -sunflower oil and the n-octane with and without an continued agitation and at two different temperatures 20 and 40°C using the method of extraction by the chloroform. It should be noted that the technique of the internal standard has been used in this study. Figures 3 and 4 represent, respectively, the chromatograms obtained and their corresponding fragmentations.

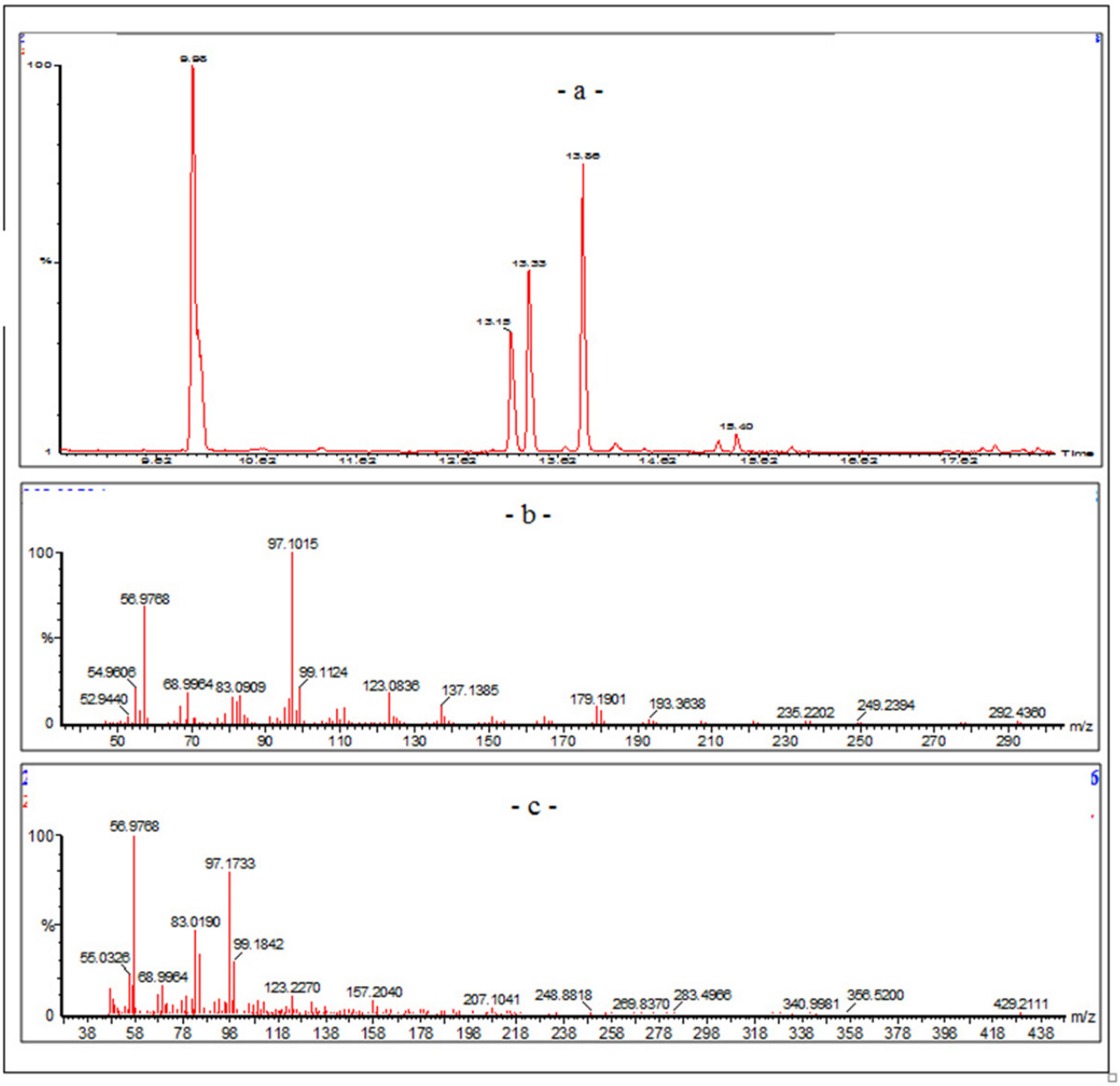


Fig. 3 **a)** Chromatogram of the sample in contact with the sunflower oil for 10 days without agitation at 20°C; **b)** Fragmentation of the peak retention time of 9.98min; **c)** Fragmentation of the peak retention time of 13.86min.

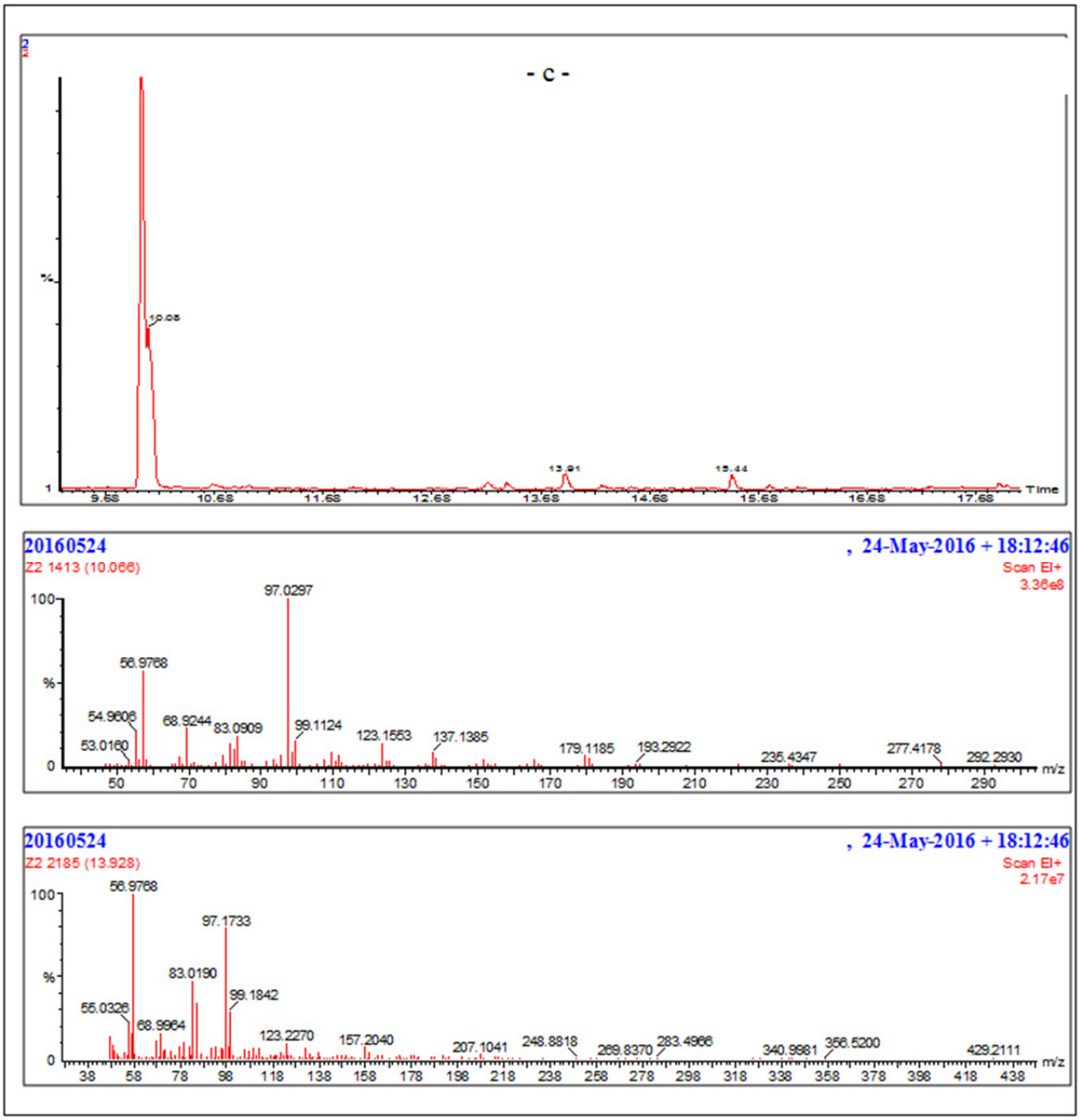


Fig. 4 **a)** Chromatogram of the sample in contact with the sunflower oil for 10 days without agitation at 40 °C; **b)** Fragmentation of the peak time retention of 10.01min; **c)** Fragmentation of the peak time retention of 13.91min.

We note the presence of two major peaks; the first has a retention time equal to 9.98 minutes with a report m/z equal to 97,10 and the second has a retention time equal to 13.86 minutes with a report m/z equal to 56.98 which correspond to the standard peak of bis 2-ethyl HexylAdipate.

From the chromatograms of the PP pellet extract and the mixed standard analyzed (Benzyl Butyl Phthalate, Bis (2-Ethyl Hexyl) Phthalate, Bis 2-Ethyl hexyl adipate, DN-Butyl Phthalate, Di-N- Octyl phthalate, diethyl phthalate, dimethyl phthalate) by GC / MS under the same operating conditions, it can be noted that the peak having the m / z ratio equal to 97 with tr = 9.98 min has substantially the same Retention of Di-Octyl Adipate (tr = 10 min). Through these spectra, it can be confirmed that the plasticizer used in the formulation of the PP pellets is Di-Octyl Adipate.

Table 5 illustrates the quantity migrated from Di-Octyle Adipat (DOA) in sunflower oil to two different temperatures, namely, 20 and 40°C.

Table 5 Migrated quantity of DOA.

Sunflower oil without agitation 20°C	Tr	9.98	13.86
	Surface	434426976	231373024
	Concentration (ppm)	$6.12*10^{-3}$	$4.23*10^{-3}$
Sunflower oil without agitation 40°C	Tr	10.01	13.91
	Surface	261922608	7731452
	Concentration (ppm)	$3.36*10^{-2}$	$2.17*10^{-1}$

From this table, we note that the areas of the peaks of samples measured in sunflower oil at 20°C are more important than those at 40°C, which means that the concentrations of DOA are more important at 20°C. In addition, the results of Table 6 highlight the influence of the temperature on the phenomenon of migration.

4. Conclusion

The monomers of the basic material or the additives incorporated in plastic can be transferred in the food, with consequences on the organoleptic plans or toxicological consequence. as the migration is a function of the affinity between the migrant and the packaged product, the phenomenon of migration depends on the composition of packaging (concentration of molecules, nature and volatility) but also that of the food.

Some of the constituents of the food are capable to get about into the packaging and change its structure; also they can activate the migration of plastic compounds. The packaging may be permeable to environmental contamination which can also alter the quality of food.

In this context, the present work had the following objectives:

- To study the interactions bottles of milk /food.
- Quantify the quantity of Di Octyl Adipate acid (DOA) migrated by the GC/MS.

In this study the phenomenon of migration to the Interfaces packaging of milk - food simulant has been studied. The migration tests have been carried out with and without agitation at 20 and 40°C in two food simulants: Sunflower oil and n-Octane.

This study has required the intervention of different techniques of analysis namely Fourier-transform infrared spectroscopy (FTIR-ATR), the atomic absorption spectrometry (AAS) and gas chromatography-mass spectrometry (GC/MS) as well an estimate of overall migration.

The applications of the FTIR spectroscopy-ATR which is a very simple technique to the analysis of samples which have undergone the tests of migration allow identifying the PP as the polymer constitutive of these bottles. The follow of the variation of the characteristics bands of this additive in function of the time of contact between the samples and food simulants has shown that a phenomenon of migration of this additives occurred.

The AAS allowed the determination of some elements attributed to the additives present in the formulation of this bottle which are: the Ca, Pb, Cd and Zn.in the samples having undergone the migration tests.

The follow of the variation of the residual of metals in the samples has highlighted the migration of additives throughout the duration of contact.

The analysis of the plastic of the selected bottle which has undergone the tests of migration by the GC/MS has confirmed what has been obtained in the study of the phenomenon of migration by the FTIR and by the AAS, because it has allowed:

- To identify the different additives contents in the formulation of the plastic used in the manufacture of these bottles which is the plasticizer (DOA)
- To clarify the migration of additives which is the DOA.
- To quantify the concentration of the DOA in the samples which have undergone the tests of migration.
- To highlight the influence of different parameters tested on the migration of additives from bottles which were in contact with the two food simulants.

Broadly, this study has therefore confirmed that the phenomenon of migration occurred to the interface bottles of milk - physiological fluid. In addition, it has shown the influence of certain parameters such as: temperature, nature of the food simulant and the agitation.

References

[1] Frawley.J.P, Scientificevidence and commonsense as a basis for food-packaging regulations, Food and Cosmetics Toxicology. Vol.5, pp 293-308, (1967).

[2] Lhugnenot.J.C, La sécurité des emballages destinés aux produits alimentaires, Industrie agroalimentaire, N° 4, pp 189-190, Avril (1996).

[3] Bohatier.C, Vernat-Rossi.V, bryesse.C, Caractérisation d'un emballage défectueux par analyse de sa fraction volatile, Industrie agro-alimentaire, pp 849-852, (1995).

[4] Durand.C, Généralités sur la perméabilité aux arômes, Industrie agroalimentaire, N° 4, PP 211-215, Avril (1996).
[5] Bureau, G. La neutralité des emballages alimentaires: les évolutions réglementaires, Ind. Alim. Agr, N°4, PP185-188. (1996).
[6] Hottchkiss, J. H. An overview of food and packaging interaction, Food Packaging Interaction, ACS symposium series. ACS Books Advisory Board, pp 1-10, (1988).
[7] Feigenbaum, A. Evaluation de la migration des matériaux plastiques au contact des aliments par des méthodes alternatives, L'emballage des denrées alimentaires de grandes consommations, Edition Technique & Documentation -Lavoisier, Paris, (1998).
[8] CENTRE (PAO), Emballages alimentaires et sécurité du consommateur, CENTRE (PAO), Septembre (1999).
[9] Q.Wang, B.K.Storm, Separation and analysis of low molecular weight plasticizers in poly (vinyl chloride) tubes, Polymer Testing N° 24, PP 290-300, (2005).
[10] Boussoum.MO, N.Belhaneche-Bensemra, Study and optimization of a soaking treatment to reduce migration from plasticized polyvinyl chloride, Journal of Applied Polymer Science, Vol. 124, 10.1002/app.35141, Wiley Periodicals, Inc. PP 1241-1248, (Avril2012).
[11] Norme ENV 1186-4, Détermination des esters méthyliques d'acides gras, (1994).
[12] Silverstein.R.M; Webster. Fx, Spectrometric identification of organic compounds, 6ème édition/P.Cm, New York (1998).

Susceptibility profile and antimicrobial multiresistance of *Staphylococcus aureus* strains from Brazilian "coalho" cheese

D.M. Santos[1], **R.O.J. Silva**[1], **S.T. Ortiz**[1], **M.R.O. Silva**[1], **S.A.C. Andrade**[2] and **E.C.L. Machado**[1]

[1] Laboratory of Food Microbiology, Academic Center of Vitória, Federal University of Pernambuco, Alto do Reservatório, s/n, Bela Vista, 55608-680, Vitória de Santo Antão, Pernambuco, Brazil
[2] Laboratory of Fruits and Vegetables, Department of Chemical Engineering, Federal Rural University of Pernambuco, Manoel de Medeiros, s/n, Dois Irmãos, 52171-900, Recife, Pernambuco, Brazil

This study aimed to determinate the susceptibility profile and antimicrobial multiresistance of *Staphylococcus aureus* strains from "coalho" cheese commercialized on Vitória de Santo Antão, Pernambuco, Brazil. The *S. aureus* strains were obtained from 06 samples of "coalho" cheese, being 03 pasteurized (Type A) and 03 unpasteurized (Type B) samples. The susceptibility antimicrobial test was performed by disk diffusion method using Erythromycin 15µg, Clindamycin 2µg, Ciprofloxacin 5µg, Gentamicin 10µg, Tetracycline 30µg, Amikacin 30µg, Penicillin G 10 UI, Chloramphenicol 30µg, Sulfazotrin 25µg and Cefoxitin 30µg as antimicrobials reference. A total of 48 strains of *S. aureus* were obtained and submitted to the antimicrobial susceptibility test. All the strains were susceptible to Ciprofloxacin, Gentamicin and Amikacin, while 24 (50%) *S. aureus* strains presented resistance to Penicillin and thus considered as penicillinase producers and and 5 (10.42%) *S. aureus* strains were resistant to cefoxitin, being considered as MRSA strains. For the Multiple Antibiotic Resistance (MAR) indexes, were observed values of 0.2, 0.3 and 0.4 correlated with the resistance to two or more antimicrobials class and 0.2 index was the most frequent. As conclusion, there was an occurrence of *S. aureus* strains with antimicrobial multiresistance in both type A and B "coalho" cheese samples, emphasizing the importance of the control of the abusive use of antibiotics by supervisory agencies.

Keywords: Antimicrobial resistance; "Coalho" cheese; *Staphylococci*; Milk derivatives

1. Introduction

The "coalho" cheese is a typical artisanal product from Brazilian Northeast that presents a great local production and consumption. Characterized by a medium to high humidity, this artisanal cheese could be easily contaminated by pathogenic microorganisms. Its trade is very widespread, being found in open-air markets, supermarkets and can be consumed in different forms: raw, roasted, fried or cooked [1].

Recently, Brazil has been showed great advances in relation to the microbiological quality of the milk produced, mainly after the implementation of the new legislation for the dairy sector [2]. However, there are still problems related to the contamination of the milk [3] and different factors are associated with the sanitary quality of milk, milk production, handling and their cooling. The contaminations that can occur in milk result in a series of changes in its physical-chemical composition, and consequently, it has a repercussion on the reduction of the quality of its derivatives, implying commercial and financial losses [4]. In order to avoid possible intoxications or food-borne diseases through consumption, the Ministry of Livestock and Food Supply determines that "coalho" cheese must be obtained from the coagulation of pasteurized milk and must be marketed with up to 10 days of manufacture [5].

Staphylococcus aureus is one of the most important human and animal pathogenic organism, being widely related with a diverse spectrum of diseases ranging from minor skin infections to life threatening diseases, such as pneumonia and meningitis, as well as, food-borne diseases, production of toxins and antimicrobial resistance [6, 7]. Antimicrobial resistance is an important health problem worldwide because the development of resistance has been associated with the extensive therapeutic use of antimicrobials or with their use as growth promoters in animal feed production. The antimicrobial multiresistant *S. aureus* strains may have an increased ability to spread, especially if they are enhanced with virulence genes. Artisanal products such as "coalho" cheese have been implicated as potential source for the transmission of the pathogen to humans. Furthermore, milk derivative products contaminated with antibiotic resistant bacteria represent ideal vehicles for the transmission of pathogenic strains [8].

In face of this perspective, the objective of this study was to evaluate the susceptibility profile and antimicrobial multiresistance of *Staphylococcus aureus* strains obtained from "coalho" cheese commercialized on Vitória de Santo Antão, Pernambuco, Brazil.

2. Material and Methods

2.1 Obtention and preparation of the "Coalho" cheese samples

For this study, the "coalho" cheese samples were collected from local and open-air markets in Vitória de Santo Antão, Pernambuco, Brazil, totaling 06 samples, being 03 pasteurized (Type A) and 03 unpasteurized (Type B). All samples

were identified, stored into thermal boxes with ice, conduced to the Laboratory of Food Microbiology of the Federal University of Pernambuco and maintained under refrigeration until microbiological analysis.

Posterly, 25 ± 0,2 g of each "coalho" cheese sample was taken and combined with 225 ml of sterile peptonated water 0.1% (w/v) in a sterile polyethylene bag and pummeled with a Stomacher during 5 min. This mixture (10^{-1}) was shaken and subsequent serial dilutions were prepared in accord with Normative Instruction Nº 62/2003 of the Ministry of Livestock and Food Supply [9]

2.2 Determination of the coagulase-positive *Staphylococci* and confirmation of *Staphylococcus aureus*

The determination of coagulase-positive *Staphylococci* was performed by *spread plate* method as described in the Normative Instruction Nº 62/2003 of the Ministry of Livestock and Food Supply [9]. A volume of 100 µl of each dilution was spreaded onto Petri plates containing Baird Parker (BP) agar supplemented with 20% egg yolk tellurite emulsion. The plates were incubated at 37°C for 24 – 48 hous. Typical and atypical colonies as circular, smooth, black or not and surrounded by an opaque zone with an outer clear zone were selected, counted and the results expressed in colony forming units (CFU/g) for "coalho" cheese sample. Further, typical and atypical colonies were transferred into tubes containing Brain Heart Infusion (BHI) broth, incubated at 37°C for 24 hours and used for the coagulase test in order to confirm coagulase-positive *Staphylococci*. The colonies that showed to be coagulase-positive were taken for *Staphylococcus aureus* confirmatory testing such as Gram stain, catalase, mannitol fermentation and DNase.

2.3 Antimicrobial susceptibility test and multiple antibiotic resistance (MAR) index

All *S. aureus* strains were used for the antimicrobial susceptibility test by disk diffusion method as CLSI M100-S25 protocol [10]. Erythromycin 15µg, Clindamycin 2µg, Ciprofloxacin 5µg, Gentamicin 10µg, Tetracycline 30µg, Amikacin 30µg, Penicillin G 10 UI, Chloramphenicol 30µg, Sulfazotrin 25µg and Cefoxitin 30µg were used as antimicrobials reference. The antimicrobial susceptibility profile was obtained by measuring the sizes of the inhibition zones and expressed in millimeters, being classified as resistant, intermediate and susceptible according to the reference table established by CLSI. The multiple antibiotic resistance (MAR) index was defined as *a*/*b*, where *a* represents the number of antibiotics to which the isolate was resistant, and *b* represents the number of antibiotics to which the isolate was exposed [11].

3. Results and Discussion

A total of 48 *Staphylococcus aureus* strains were obtained from "coalho cheese" samples and submitted to the antimicrobial susceptibility test, being 15 (31.25%) from pasteurized cheese (Type A) and 33 (68.75%) from unpasteurized cheese (Type B). The antimicrobial susceptibility profile showed that 14 (29.16%) *S. aureus* strains were susceptible to all antimicrobials tested and 34 (70.84%) were resistante to at least one antimicrobial. The Table 1 contains the results for antimicrobial susceptibility profile of each antimicrobial related to 48 *S. aureus* strains. Gentamicin, amikacin and ciprofloxacin were effective towards all the *S. aureus* strains (100% susceptible).

A study carried out with 45 *S. aureus* strains from 10 rennet cheese samples showed a high percentage of resistance to penicillin (100%), tetracycline (75.5%), gentamicin (66.7%), erythromycin (48.9%) and sulfazotrin (26.7%) [12].

Table 1 Antimicrobial resistance percentage for the 48 *Staphylococcus aureus* strains obtained from "coalho" cheese commercialized on Vitória de Santo Antão, PE.

Antimicrobial Agents	Resistant	Susceptible
Erythromycin	11 (22.91%)	37 (77.08%)
Penicillin	24 (50%)	24 (50%)
Clindamycin	11 (22.91%)	37 (77.08%)
Ciprofloxacin	0	48 (100%)
Gentamicin	0	48 (100%)
Cefoxitin	5 (10.42%)	43 (89.58%)
Tetracycline	12 (25%)	36 (75%)
Amikacin	0	48 (100%)
Sulfazotrin	1 (2.08%)	47 (97.91%)
Chloramphenicol	3 (6.25%)	45 (93.75%)

Considering the main resistance phenotypes, our results pointed that 24 (50%) *S. aureus* strains were resistant to penicillin and thus considered as penicillinase producers and 5 (10.42%) *S. aureus* strains, isolated from unpasteurized cheese (Type B), were resistant to cefoxitin and considered as MRSA strains. The CLSI has suggested the usefulness of the cefoxitin disk instead of the oxacillin disk when used as a surrogate marker for the detection of methicillin resistance. In recent years, an increase in the number of methicillin resistant (MR) *Staphylococcus aureus* strains has

become a serious clinical and epidemiological problem, as resistance to this antibiotic implies resistance to all β-lactam antibiotics, including carbapenems. Methicillin resistance in *S. aureus* is mediated through an protein called low-affinity penicillin binding protein (PBP2a) and encoded by *mec*A gene [13].

MLS (Macrolide-Lincosamide-Streptogramin) resistance phenotype was also observed between *Staphylococcus aureus* strains. Resistance to erythromycin as well as to clindamycin was observed in 7 (14.6%) *S. aureus* strains indicated a constitutive type of MLS resistance (cMLS) while 4 (8.33%) *S. aureus* strains showed resistance to erythromycin and being susceptible to clindamycin, were classified only as MS resistance phenotype.

MAR index is a very useful tool to understand the risks associated with to antimicrobial multiresistance. The Table 2 shows the values for the total of 48 *S. aureus* strains. As observed, the most of the *S. aureus* strains presented index of 0.2, which mean resistance towards two antimicrobials of different classes.

Table 2 Percentage of 48 *Staphylococcus aureus* strains in according to the multiple antibiotic resistance (MAR) index.

MAR Index	***S. aureus* strains**
0.1	12 (25%)
0.2	13 (27.1%)
0.3	7 (14.6%)
0.4	2 (4.1%)

Special attention should be given to the MAR index 0.4, since what 2 (4.1%) *S. aureus* strains showed resistance to 4 antimicrobial agents of different classes as macrolide, lincosamide, tetracycline and β-lactams. These strains were isolated from unpasteurized cheese (Type B), showing the great importance of the pasteurization process in elimination of pathogens and the risk of food contamination by resistant bacteria.

It was observed the occurrence of distinct antimicrobial resistance profile between the *S. aureus* strains of "coalho" cheese produced by pasteurized milk (type A) and unpasteurized (Type B), a percentage of 80% corresponding to 12 *S. aureus* strains and a percentage of 66% corresponding to 22 *S. aureus* strains as shown in Table 3, respectively.

Table 3 Percentage of antimicrobial resistance for *Staphylococcus aureus* strains related with the "coalho" cheese type.

"Coalho" Cheese	***S. aureus* Strains Resistance (%)**
Type A (pasteurized)	12/15 (80%)
Type B (unpasteurized)	22/33 (66%)

Staphylococcus aureus is one of the most common microorganisms associated with food poisoning in the world, being a pathogen frequently isolated in artisanal cheeses [14, 15] and used as an indicator of post-process contamination production or hygienic-sanitary conditions of the commercial places [16].

Due to the high antimicrobial multiresistance presented by *S. aureus* strains isolated from "coalho" cheese (Type A and B) and mainly because some of these strains showing interesting resistance phenotypes such as MRSA and cMLS, this study emphasizes the importance of control in the indiscriminate use of antibiotics to avoid problems of resistance acquired by *S. aureus* compromising the quality of milk and derived products contaminated by these strains.

Acknowledgements The authors would like to thank the Institutional Scientific Initiation Scholarship Program of the Federal University of Pernambuco (PIBIC-UFPE) and the National Council for Scientific and Technological Development (CNPq) for financial support.

References

[1] Almeida SL, Júnior PGF, Guerra JRF. A estratégia de internacionalização de negócios na perspectiva da tradução cultural: o caso da indicação geográfica no agronegócio. RIAE - Rev. Ibero-Americana de Estratégia. 2013; 9:74-97.

[2] BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa Nº 22 de 24 de novembro de 2005. Regulamento Técnico para Rotulagem de produto de Origem Animal Embalado. Diário Oficial da República Federativa do Brasil, Brasília, 25 de novembro , Seção I, 2005.

[3] Feitosa T, Borges MF, Nassu RT, Azevedo EHF, Muniz CR. Pesquisa de *Salmonella* sp., *Listeria* sp. e microrganismos indicadores higiênico-sanitários em queijos produzidos no Estado do Rio Grande do Norte. Ciênc. Tecnol. Aliment. 2003; 23:162-165.

[4] Andrade UVC, Hartmann W, Masson ML. Isolamento microbiológico, contagem de células somáticas e contagem bacteriana total em amostras de leite. Acervo Veterinária. 2010; 25:129-135.

[5] BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa Nº 30 de 26 de junho de 2001. Regulamento técnico de identidade e qualidade de manteiga da terra ou manteiga de garrafa; queijo de coalho e queijo de manteiga. Diário Oficial da República Federativa do Brasil, Brasília, 16 de julho de 200, Seção I, 2001.

[6] Ferreira MA, Bernardo LG, Neves LS, Campos MR, Lamaro-Cardoso J, André MC. Virulence profile and genetic variability of *Staphylococcus aureus* isolated from artisanal cheese. J. Dairy Sci, 2016; 99:8589–8597.

[7] Sasidharan S, Prema B, Yoga Latha L. Antimicrobial drug resistance of *Staphylococcus aureus* in dairy products. Asian Pac J Trop Biomed. 2011; 1: 130-132.

[8] Akindolire MA, Babalola OO, Ateba CN. Detection of Antibiotic Resistant *Staphylococcus aureus* from Milk: A Public Health Implication. Int J Environ Res Public Health. 2015;12: 10254–10275.
[9] BRASIL. Ministério da Agricultura. Instrução Normativa Nº62, de 26 de agosto de 2003. Oficialização os métodos analíticos oficiais para análises microbiológicas e para controle de produtos de origem animal e água. Brasília, DF, 2003.
[10] CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement. CLSI document M100-S25. Wayne, PA: Clinical and Laboratory Standards Institute; 2015.
[11] Krumperman PH. Multiple Antibiotic Resistance Indexing of *Escherichia coli* to Identify High-Risk Sources of Fecal Contamination of Foods. Applied and Environmental Microbiology. 1983; 165-170.
[12] Araujo JG, Alves EMG, Bechtluff MP. Perfil de dna plasmidial em bactérias resistentes a antibióticos isoladas do Ribeirão Paciência – Pará de Minas- MG. 2009; 1:282-292.
[13] Jain A, Agarwal A, Verma RK. Cefoxitin disc diffusion test for detection of meticillin-resistant *Staphylococci*. Journal of Medical Microbiology. 2008; 57:957–961.
[14] Acco M, Ferreira FS, Henriques JAP, Tondo EC. Identification of multiple strains of *Staphylococcus aureus* colonizing nasal mucosa of food handlers. Food Microbiology. 2003; 20: 489-493.
[15] Kuchenbecker BS, Ribeiro AR, Cardoso M. Perfil de resistência de isolados de *Staphylococcus aureus* obtidos de produtos de origem animal analisados pelo Serviço de Inspeção Federal do Brasil. Acta Scientiae Veterinariae. 2008; 2:143-149.
[16] Silva N, Junqueira VCA, Silveira NFA. Manual de métodos de análises microbiológica de alimentos. São Paulo: Livraria Varella, 1997.

Tackling food and nutrition insecurity using leafy wild vegetables: The nutritional compositions of some selected species

C. Bvenura and **A.J. Afolayan**

Medicinal Plants and Economic Development (MPED) Research Center, University of Fort Hare, P Bag X1314, Alice, 5700, South Africa

Fourteen wild vegetable species were analysed to determine their proximate compositions and mineral constituents. Fibre crude content ranged between 0.39 and 1.79 g/100 g; crude protein between 0.48 and 1.53 g/100 g; crude lipid between 0.02 and 3.83 g/100 g; phytate between 0.92 and 8.92 g/100 g and ash content was between 0.39 and 1.79 g/100 g. *Solanum nigrum*, *Tulbaghia violacea*, *Chenopodium album* and *Chenopodium murale* had the highest concentrations of fibre, protein, lipid, phytate and ash respectively. Calcium, magnesium, potassium, sodium, phosphorus, copper, iron, zinc, manganese and vitamin C ranged between 6.70-34.84; 1.54-22.79; 50.6-125.97; 0.25-18.73; 2.10-4.76; 0.01-.0.02; 0.21-2.60; 0.12-0.60; 0.04-0.60 and 41.67-225.00 g/100 g respectively. *Chenopodium murale* had the highest concentration of Mg, K and P while *Physalis peruviana* had the highest concentration of Fe and vitamin C. Copper was remarkably low in all the wild vegetables. This study revealed the potential of wild vegetables to meet the daily requirements of nutrients needed for human health. The nutritional content suggests that inclusion of these vegetables in the diet may help alleviate hunger and nutritional deficiency in the Eastern Cape by enhancing the function and nutritional properties of food and food products. Also, the supplementing the diet with these nutrient rich foods is in line with global and government programmes aimed at drastically reducing food and nutrition insecurity.

Keywords: Mineral; proximate; wild vegetables; nutrition; anti-nutrient; food security

1. Introduction

Wild vegetables have been a part of the human diet since the beginning of time. Human consumption of these vegetables and the ability of the species to meet nutrient needs have been documented for a long time. It is estimated that, at least, one billion people globally still include wild foods in their diets (Aberoumand and Deokule, 2010). In South Africa, Wehmeyer and Rose (1983) identified more than 100 plant species that are being used as wild vegetables.

In many places of the developing world, there has been a low trend in the consumption of wild vegetables. This reduction in the consumption of wild vegetables has been attributed to seasonal availability and culture in Nigeria, while in South Africa, culture, taste and affordability have been cited as the major causes (WHO, 2003; Hart et al., 2005; Shackelton et al., 2009; Faber et al., 2010). Wild vegetables are usually consumed by the rural populace as supplements to their diet since the vegetables usually naturally grow on cultivated or fallow fields thereby making them easily accessible. However, the urban populace pay less attention to wild vegetables in favour of the conventional ones because of easy accessibility of the later. The use of herbicides, pesticides, as well as excessive cultivation of the fields has led to the decline in the availability of wild vegetables and subsequent decline in their knowledge (Odhav et al., 2007). Also, the perception, especially among young people, that such vegetables are foods for the poor, causes lack of interest in the cultivation and nutritional importance of these plants (Vorster et al., 2007).

Yet studies have revealed that wild vegetables have numerous beneficial nutritional values often better than the domesticated exotic breeds like spinach and cabbage (Odhav et al., 2007; Lewu and Mavengahama, 2010). The use of wild vegetables could be lost with time if their knowledge, especially on the identification, nutrient value, methods of preparation and preservation are not passed down to the younger generations and properly documented. Therefore, this study was conducted to investigate the nutritional compositions of some of the wild vegetables growing in the Eastern Cape Province of South Africa, in an effort to create an awareness of some important aspects of these neglected food plants. The inclusion of these vegetables in the diet could help alleviate food and nutrition security concerns being faced by the inhabitants of the province.

2. Materials and methods

2.1 Plant collection and preparation

Fourteen, wild vegetables were collected fresh from the Mbashe and Nkonkobe Municipalities of the Eastern Cape during the rainy season when the wild vegetables were vegetatively growing in home gardens and the fields. The plants were initially identified by their vernacular names (Xhosa) and later validated at the

University of Fort Hare Herbarium (Bvenura and Afolayan, 2014). Young and tender shoots were plucked from the mother plant as practised locally, stored in khaki paper sampling bags and transported to the laboratory where they were washed thoroughly with distilled water. Leaves of the same plant from the two municipalities were combined to make a single composite sample, oven dried at 40°C to a constant weight and homogenised using a 2 mm sieve Polymix (PX-MFC 90 D) electric grinder, after which they were sealed in polyethylene bags and stored in the refrigerator at 4°C until needed for the various analysis.

2.2 Proximate analysis

2.2.1 Determination of ash content

About 5 g of the powdered plant leaf sample was weighed into a previously weighed crucible. This was incinerated in an E-Range muffle furnace with TOHO P4 programme at 550°C for 12 h. The final weight of the sample was used to calculate the ash content as follows:

Ash content (%) =$(final\ weight\ of\ sample\ after\ incineration\ (g))\ /\ (5\ g)$ x 100 % (Antia et al, 2006).

2.2.2 Determination of crude lipid

Crude lipid was determined as described by Antia et al. (2006). About 5 g of the powdered sample was measured into a 250 ml beaker, 100 ml of diethyl ether was added, covered with aluminium foil and shaken in an orbital shaker for 24 h. Filtration followed this process and the supernatant was decanted. Another 100 ml of diethyl ether was added to the residue and shaken for another 24 h. The residue obtained after filtration was the lipid free sample and was calculated as:

$$\text{Crude lipid} = \frac{Weight\ of\ sample\ after\ diethyl\ ether\ extraction}{Initial\ weight\ of\ sample} \text{ x } 100\ \%$$

2.2.3 Determination of crude fiber

The AOAC (1984) method was used in estimating the crude fibre. About 5 g of the powdered sample was weighed into a beaker and digested in 100 ml of 1.25 % sulphuric acid for 30 min. The acid digested sample was allowed to cool, and then filtered. The residue was collected into a beaker and further digested in 100 ml of 1.25 % sodium hydroxide. The sample was filtered and the residue dried in an oven at 100°C to a constant weight. The dried residue was then incinerated in a muffle furnace for 24 h at 550°C. The crude fiber was obtained from the loss in weight on ignition of dried residue remaining after digestion of fat free samples (AOAC, 1984):

$$\%\ \text{fiber} = \frac{Loss\ of\ weight\ on\ ignition}{Weight\ of\ sample\ used} \text{ x } 100\ \%$$

2.2.4 Determination of vitamin C

Preparation of iodine solution

The iodometric titration method was used to determine the vitamin C content. About 5 g of potassium iodide and 0.268 g potassium iodate were dissolved in 200 ml distilled water in a 400 ml beaker, followed by addition of 30 ml of 3 M sulphuric acid. The mixture was poured into a 500 ml graduated cylinder and then diluted to a final volume of 500 ml with distilled water. Vitamin C standard solution was prepared by dissolving 0.250 g vitamin C in 100 ml water. This was made up to 250 ml with distilled water.

Standardisation of iodine with Vitamin C standard solution

About 25 ml of vitamin C standard solution was measured into a 125 ml Erlenmeyer flask, following which 10 drops of 1 % starch solution were added as the indicator. This was titrated against the acidified potassium iodide iodine solution until the end point (the first blue colour that showed after at least 20 s of swirling) was reached.

Vitamin C determination in the samples

About 5 g of fresh leaf samples were macerated in 20 ml of distilled water. The mixture was filtered and the filtrate collected in a 50 ml volumetric flask and made to the mark with distilled water. About 10 ml of the sample solution was transferred into an Erlenmeyer flask and 10 drops of 1 % starch added and titrated against the acidified potassium iodide solution.

2.2.5 Determination of phytate components

Phytate was determined according to the method of Wheeler and Ferrel (1971). A sample of finely ground plant material measuring 4 g was soaked in 100 ml of 2 % hydrochloric acid for 3 h and then filtered through Whatman No. 43 filter paper. About 25 ml of the filtrate was measured into a conical flask and 5 ml of 0.3 % ammonium thiocyanate solution was added as indicator, followed by addition of 53.5 ml distilled water. This was titrated against a 1000 ppm standard iron (III) chloride solution until a brownish yellow colour persisted for 5 min. The Phytate content was calculated from the iron determinations, assuming a 4:6 iron to Phytate molecular ratios and multiplied by a constant of 3.55 (Vijayakumari et al., 1996).

2.2.6 Determination of protein

About 0.5 g of finely ground vegetable samples was placed in dry, clean digestion tubes and 5 ml of the digestion mixture comprising 1 part $HClO_4$ + 2 parts HNO_3 added. This mixture was digested at 230^{o}C on a digestion block for 70 min, allowed to cool down and made up to 100 ml volume with distilled water. The concentration of nitrogen was then determined using the Inductively Coupled Plasma - Optical Emission Spectrometer (ICP OES). Percentage crude protein was obtained by multiplying the nitrogen value by a factor of 6.25 (AGRILASA, 2008).

2.3 Mineral analysis

Macro-minerals (Phosphorus, Potassium, Calcium, sodium and Magnesium) and micro-minerals (Copper, Iron, Zinc and Manganese) were determined using the method described above for the determination of nitrogen.

2.4 Statistical analysis

Data of the nutrient concentrations of various wild vegetables were subjected to statistical analysis using MNITAB Release 12. A one-way analysis of variance was used to compare the means of various nutrient concentrations among the wild vegetables. Means were segregated using Duncan's multiple range test. The means were treated as significantly different at $p < 0.05$.

3. Results

3.1 Proximate composition

The proximate compositions of the wild vegetables gathered from Mbashe and Nkonkobe municipalities in the Eastern Cape Province of South Africa varied significantly ($p < 0.05$) among the vegetables (Table 1). Crude fibre ranged between 0.91 and 6.79 g/100g; crude protein between 0.47 and 1.53 g/100g; crude lipid between 0.14 and 3.83 g/100g; phytate between 1.09 and 8.92 g/100g and ash content was between 0.38 and 1.79 g/100g. *Tulbaghia violacea* had the highest protein and *Sonchus oleraceus* the highest lipid content. The lowest protein content was recorded in *Chenopodium murale* while the highest fibre and ash contents were recorded in *Solanum nigrum* and *Chenopodium murale* respectively. The lowest ash values were observed in *Solanum nigrum*.

3.2 Mineral composition

The mineral compositions of the 14 vegetables significantly differed among the vegetables as shown in Table 2. The mean concentration of nutrients in all the vegetables decreased in the order Vit C > K > Ca > Mg > Na > P > Fe > Zn > Mn > Cu. *Chenopodium murale* contained the highest K, Mg and P, while *Physalis peruviana* contained the highest concentration of Fe and Vit C. Cu was remarkably low in all the wild vegetables.

Table 1 Proximate compositions (g/100 g) of 14 vegetables in Mbashe and Nkonkobe Municipalities in the Eastern Cape Province of South Africa .

Vegetable species	†Phytate	Crude protein	Crude lipid	Crude fibre	Ash
Bidens pilosa L.	2.4±6.51^{c}	1.19±0.03^{ab}	1.60±0.22^{d}	2.14±0.32^{c}	0.38±0.03^{b}
Centella coriacea Nannfd.	2.31±1.31^{a}	1.1±0.05^{ab}	0.52±0.02^{b}	1.09±0.03^{a}	1.74±0.05^{c}
Chenopodium album L.	8.92±0.38^{a}	0.48±0.01^{a}	1.15±0.19^{b}	1.68±0.02^{ac}	1.38±0.04^{d}
Chenopodium murale L.	3.07±4.30^{b}	0.47±0.01^{a}	3.50±0.03^{a}	1.35±0.01^{a}	1.79±0.02^{c}
Cotula heterocarpa DC.	2.23±0.57^{ab}	1.02±0.14^{ab}	0.84±0.05^{b}	1.01±0.02^{a}	1.44±0.01^{d}
Galinsoga parviflora Cav.	2.98±0.81^{c}	0.94±0.08^{ab}	2.51±0.46^{e}	1.66±0.29^{c}	0.45±0.03^{b}
Hypochaeris radicata L.	1.09±0.81^{a}	0.82±0.07^{ab}	0.14±0.03^{f}	1.00±0.01^{a}	0.80±0.16^{a}
Physalis peruviana L.	4.19±0.85^{ab}	1.02±0.14^{ab}	1.93±0.51^{de}	1.53±0.03^{ac}	1.39±0.24^{d}
Rumex obtusifolius L.	4.86±1.05^{a}	0.48±0.01^{a}	0.35±0.11^{c}	1.08±0.01^{a}	1.24±0.01^{d}
Solunum nigrum L.	2.34±0.50^{a}	0.56±0.02^{a}	0.58±0.02^{b}	6.79±0.01^{b}	0.39±0.02^{b}
Sonchus oleraceus L.	1.50±5.37^{d}	1.09±0.06^{ab}	3.83±0.04^{a}	1.11±0.02^{a}	0.77±0.05^{a}
Stellaria media L.	4.64±0.61^{b}	0.51±0.00^{a}	2.56±0.06^{e}	1.26±0.01^{a}	1.55±0.01^{d}
Tulbaghia violacea Harv.	1.55±2.14^{c}	1.53±0.13^{ab}	0.64±0.04^{b}	0.91±0.04^{a}	1.29±0.0.02^{d}
Urtica urens L.	2.51±0.90^{a}	0.87±0.13^{ab}	0.64±0.02^{b}	2.35±0.02^{c}	1.67±0.0.01^{cd}

Different letters down the same column represent significant differences at $p < 0.05$.

Table 2 Mineral compositions (g/100 g) of 14 vegetables in Mbashe and Nkonkobe Municipalities in the Eastern Cape Province of South Africa.

Vegetable species	Mineral Element									
	Ca	Mg	K	Na	P	Cu	Fe	Zn	Mn	Vitamin C
B. pilosa	14.70±3.48^{e}	3.46±1.51^{cb}	71.95±74.00^{cd}	0.28±1.99^{a}	2.57±0.67^{f}	0.01±0.01	0.87±0.53^{c}	0.28±2.73^{b}	0.13±0.06^{ab}	0.042±0.06^{a}
C. album	14.39±1.33^{e}	16.69±6.93^{f}	110.88±54.30^{fg}	2.54±0.98^{c}	4.29±0.11^{g}	0.01±0.00	0.26±0.19^{a}	0.15±3.76^{a}	0.15±0.04^{ab}	0.125±0.06^{a}
C. coriacea	10.40±2.70^{cd}	2.69±0.20^{cb}	16.80±21.50^{a}	22.01±1.86^{g}	2.10±0.19^{a}	0.01±0.03	0.68±0.19^{b}	0.12±0.18^{a}	0.21±1.80^{b}	0.050±0.03^{a}
C. heterocarpa	7.27±4.66^{ab}	2.72±0.66^{b}	61.65±29.40^{c}	12.44±2.78^{e}	3.56±0.26^{e}	0.01±0.01	0.76±0.40^{bc}	0.16±0.31^{a}	0.07±0.03^{a}	0.058±0.06^{a}
C. murale	11.34±4.26^{d}	22.79±19.64^{h}	125.97±4.20^{g}	5.37±2.14^{cd}	4.76±0.56^{i}	0.01±0.00	0.59±0.73^{a}	0.14±0.23^{a}	0.05±0.51^{a}	0.117±0.00^{b}
G. parviflora	24.77±3.41^{g}	4.04±1.51^{c}	53.31±11 4.00^{b}	0.38±1.13^{a}	4.51±0.74^{h}	0.01±0.01	0.53±0.07^{b}	0.14±0.30^{a}	0.13±0.08^{ab}	0.047±0.15^{d}
H. radicata	9.14±6.80^{c}	4.10±0.17^{c}	52.00±11.10^{b}	19.54±6.00^{f}	2.57±0.55^{b}	0.01±0.02	0.42±0.15^{b}	0.28±0.22^{b}	0.07±0.06^{a}	0.048±0.02^{a}
P. peruviana	13.11±7.50^{e}	9.70±7.21^{e}	84.63±85.20^{cd}	0.25±2.82^{a}	2.89±0.91^{c}	0.02±0.01	2.60±2.29^{e}	0.34±0.22^{c}	0.10±0.49^{a}	0.225±0.03^{a}
R. obtusifolius	8.03±3.59^{b}	4.93±1.56^{c}	73.28±63.10^{c}	0.86±0.65^{a}	4.59±0.71^{h}	0.01±0.01	0.44±0.80^{ab}	0.14±0.29^{a}	0.06±0.85^{a}	0.108±0.10^{c}
S. media	6.70±6.98^{a}	6.05±1.56^{d}	121.52±73.80^{g}	3.83±1.79^{c}	4.43±1.18^{g}	0.01±0.00	0.77±0.48^{cb}	0.30±3.91^{bc}	0.08±0.29^{a}	0.042±0.10^{a}
S. nigrum	16.98±4.59^{f}	3.72±1.46^{cb}	76.91±80.40^{d}	0.65±1.09^{ab}	3.88±0.70^{f}	0.01±0.06	0.43±0.66^{b}	0.12±0.50^{a}	0.07±0.02^{a}	0.014±0.04^{a}
S. oleraceus	12.25±4.78^{de}	4.15±0.54^{c}	50.6±5.20^{b}	18.73±2.37^{f}	3.17±0.74^{d}	0.02±.03	0.56±0.59^{b}	0.14±0.39^{a}	0.04±0.41^{a}	0.058±0.15^{b}
T. violacea	7.36±3.98^{ab}	1.54±0.80^{a}	80.44±38.70^{cd}	3.72±2.39^{c}	3.65±1.54^{e}	0.01±0.01	0.21±0.19^{a}	0.19±0.48^{a}	0.02±0.02^{a}	0.162±0.06^{b}
U. urens	34.84±3.65^{i}	4.25±1.58^{c}	66.99±12.90^{c}	0.41±1.20^{a}	4.68±0.56^{ih}	0.01±0.01	0.35±0.17^{ab}	0.16±0.47^{a}	0.10±0.04^{a}	0.045±0.02^{a}

Different letters down the same column represent significant differences at $p < 0.05$

4. Discussion

4.1 Proximate composition

The proximate analysis revealed that all the 14 wild vegetables are rich in the various nutrients investigated but in varying proportions. Ash, protein, lipid and fibre contents in *Sonchus oleraceus* were lower than earlier reported by Jimoh et al. (2011). Odhav et al. (2007) investigated the proximate composition of 20 wild plants and reported higher values for protein (3 - 7 g/100 g) and ash (1.74-4.91 g/100 g), but lipid (0.2 - 2.7 g/100 g) and fibre (1.21 - 2.92 g/100 g) were within the range of the present study. *Sonchus oleraceus* and *Chenopodium murale* showed appreciable lipid contents in this study. Lyimo et al., (2003) reported a lipid range of between 0.1 and 1.0 g/100 g in 30 wild vegetables, but lower than observed in the current study. According to Antia et al. (2006); lipids increase the palatability of food by absorbing and retaining flavours. A diet providing 1 - 2 % of its calorific energy as fat is said to be sufficient for humans, as excess fat consumption is implicated in cardiovascular disorders such as atherosclerosis, cancer and aging (Sharma et al., 2012). The considerable amount of lipids in some of these vegetables in relation to the NHMRC (2005) values would therefore improve the palatability of the vegetables and reduce the risk of some diseases. Lyimo et al. (2003) reported a protein range between of 0.6 and 5.0 g/ 100 g in 30 wild vegetables, again this was higher than the current study with a range of between 0.47 and 1.53 g/100 g. Protein is essential for growth and repair of muscles, bones, skin, tendons, ligaments, hair and eyes amongst other tissues in humans. The protein values from this study indicate that the wild vegetables have the ability to supply a fraction of the Recommended Daily Intake (RDI) values

(NHMRC, 2005). Children need to consume at least 150 g of vegetables per day to harness the required nutrients (NHMRC, 2003). For example; *Sonchus oleraceus, Tulbaghia violacea and Bidens pilosa* can supply 5.0, 5.5 and 7.1 % respectively of children's RDI values. High ash content in plants is a reflection of high mineral content (Aberoumand and Deokule, 2010). The high ash content of *Chenopodium murale* compared to other vegetables in this study is an indication of high mineral value of the vegetable. High fibre diets have been linked with lower serum cholesterol concentrations, lower risk of coronary heart disease, reduced blood pressure, enhanced weight control, reduced risk of certain forms of cancer and an improved gastrointestinal function (Anderson et al., 1994). *Solanum nigrum, Urtica urens and Bidens pilosa* can supply about 67.9, 23.5 and 21.4 % of the required daily amount in men if a quantity of about 300 g is consumed. The concentration of fibre would also presumably not lead to high fibre related conditions and diseases. Phytate is an antinutrient which has a strong ability to chelate multivalent metal ions especially Zn, Ca and Fe, leading to their poor bioavailability (Gupta et al., 2006). Although the presence of phytate could decrease mineral absorption in humans, this antinutrient is said to be heat labile (Akwaowa et al., 2000). It is therefore conceivable that the high phytate content in *Chenopodium album* (8.92 g/100 g) and *Physalis peruviana* (4.19 g/100 g) will be significantly reduced during cooking. Additionally, leafy vegetables are generally considered to be superior sources of mineral supplements and therefore would ideally lower the effect antinutrients would have on the availability of some nutrients (Odhav et al., 2007).

4.2 Mineral and vitamin C composition

Vitamin C was remarkably high in *Physalis peruviana* (0.225 g/100 g) which makes the vegetable a better source of the vitamin C compared to guava (0.188 g) and orange (0.07 g) fruits as well as some leafy vegetables such as broccoli (0.039 g) (Brand et al., 1982). The results of the current study are comparable with what was reported by Lyimo et al. (2003) in a study of 30 wild vegetables in Tanzania. These authors found that *Galinsoga parviflora*, *Bidens pilosa,* and *Solunum nigrum* respectively contained 0.054, 0.059 and 0.234 g/ 100 g vitamin C. *Sonchus oleraceus* which had the lowest vitamin C concentration has the potential to supply about 31 % of vitamin C RDI while *Physalis peruviana* which had the highest concentration can supply about 500 % of the required vitamin C RDI in adults. Vitamin C prevents tissue damage, aids in the recovery of several ailments and diseases including colds, cough, influenza, sores, wounds and skin diseases among others (Ogunlesi et al., 2010). According to Lopez and Martos (2004), vitamin C improves Fe availability, therefore reducing the risk of iron deficiency anaemia. Potassium was also remarkably high in the wild vegetables while Cu was low in the present study. In studies previously conducted by Bvenura and Afolayan (2012) in Nkonkobe Municipality, Cu was low in cabbage, spinach and carrot. The previous and current results possibly indicate the low levels of the mineral in the soil of the study area. Jimoh et al. (2011) and Kawada et al. (2002) reported slightly lower mineral values compared to the present study except for Mn, Cu and Zn, which were slightly higher. In Iran, lower levels of mineral nutrients were found in some wild vegetables as compared to the present study (Aberoumand and Deokule 2010). Research done in Akure, Nigeria indicated a slightly higher concentration of mineral nutrients compared to this study (Aletor et al., 2002). In KwaZulu Natal, South Africa, Odhav et al. (2007) found high levels of Ca, P and Mg with many wild vegetables exceeding 1000 mg/100 g. Variations in the nutrient compositions of edible plants are influenced by various factors including farming practices, prevailing environmental conditions including soil manipulation using organic and inorganic fertilisers and the age of the plants at harvest (Nordeide et al., 1996). In addition, some minerals such as Zn decrease with advancing plant age while other minerals such as Fe and Mn reportedly increase with increasing plant age (Tiffin, 1971). In this study, vegetables were collected from fields where the soil has been manipulated by addition of organic and mineral fertilisers and only young fresh plants were collected. These factors may have contributed to the amount of minerals in the vegetables. Findings of this study also indicated that all vegetable samples had Na: K ratios of less than 1. Yang et al. (2011) linked a high Na: K ratio with increased risk of cardiovascular diseases leading to mortality. These authors further reported that a high Na intake and a low K intake are linked to high blood pressure. The consumption of these vegetables would therefore not only help lower blood pressure especially among the elderly but also boost their immune system. In sub-Saharan Africa, South Africa has the 4th highest number of people living with HIV/AIDS with an estimated 5.6 million (17.8 %) people infected. The Eastern Cape Province is the 6th most affected with 9 % of the population living with the virus (UN/AIDS, 2010). Researchers around the world have strongly linked HIV to nutrition; they view nutrition as a fundamental intervention in boosting the immune system of the infected in the early stages and ongoing treatment of the disease (Elbein, 1995; Tinnerello, 1998; Charles, 2009). Among other nutrients, people living with HIV are more susceptible to low levels of Zn and Fe in their blood (Barnett, 2006). As shown by the appreciable amounts of these minerals in this study, a wild vegetable inclusive diet for example comprising *T. violacea*, *S. oleraceus* and *S. nigrum* would presumably improve the nutrition of HIV/AIDS patients. The high levels of P observed in vegetables from this study may also increase the mineral's availability in human nutrition. Mn is an activator and constituent of several enzymes and occurs in very low quantities in humans though this mineral's importance cannot be overlooked (Medeiros and Wildman, 2000). The Mn content observed in this study, though low, may supplement its presence in the diet.

In humans, Mg is a critical co-factor in more than 300 enzymatic reactions in the body while in plants the most recognised role of the element is in photosynthesis, where the element must be incorporated into the chlorophyll molecule before chlorophyll is effective at gathering light for photosynthetic carbon reduction reactions (Schachter,

2012; Wilkinson et al., 1990). Therefore, the abundance of Mg in wild vegetables is essential in ensuring a healthy plant and the subsequent availability of other minerals and their supply in the human diet. In relation to the RDI values (Table 3.2b) and the NMHRC (2003) recommended quantities for children, the mineral elements can be sufficiently supplied per 150 g cooked portion. However, one of the drawbacks with wild vegetables is their seasonal availability. These nutritionally rich foods are usually available during rainy season but this shortfall can be overcome by gathering in large quantities when available, drying and storing for off-season consumption. Furthermore, a more sustainable solution that may ensure a continuous fresh supply of these wild foods is to cultivate them in home gardens.

5. Conclusion

The results of this study reveal that the leaves of the 14 wild vegetables are rich in minerals. However, the vegetables differ in nutrient contents. However, *Sonchus oleraceus* which had the lowest vitamin C concentration has the potential to supply about 31 % of vitamin C RDI while *Physalis peruviana* which had the highest concentration can supply about 500 % of the required vitamin C RDI in adults per 300 g of serving. *Solanum nigrum, Urtica urens and Bidens pilosa* can supply about 67.9, 23.5 and 21.4 % of the required daily amount of fibre in men per 300 g serving. *Sonchus oleraceus, Tulbaghia violacea and Bidens pilosa* can supply 5.0, 5.5 and 7.1 % respectively of children's RDI values for protein per 150 g of serving. When compared to other vegetables *Chenopodium murale* is a good source of Mg, K and P while *Physalis peruviana* is a good source of Cu and Fe. Furthermore, *Physalis peruviana* is a good source of Vit C as well as protein and *Solanum nigrum* is a good source of fibre. Mixing these vegetables when cooking them as is practised locally has the potential to meet the human requirements of nutrients for growth and development on a daily basis which in turn helps to overcome nutritional deficiency problems which are prevalent especially in poor rural areas. These vegetables are therefore recommended for consumption alone or in combination with other vegetables.

References

[1] Aberoumand A, Deokule SS. Preliminary studies on proximate and mineral composition of Marchubeh stem (*Asparagus officinalis*) vegetable consumed in the Behbahan of Iran. World Journal of Dairy & Food Sciences. 2010; 9(2): 127-130.

[2] AGRILASA. Agri Laboratory Association of Southern Africa. Method 6.1.2: Wet ashing. Plant and feed analysis handbook. 2008.

[3] Akwaowa EU, Ndon BA, Etuk EU. Minerals and antinutrients in fluted pumpkin (*Telfairia occidentalis* Hook f.). Food Chemistry. 2000; 70: 235-240.

[4] Aletor O, Oshodi AA, Ipinmoroti K. Chemical composition of common leafy vegetables and functional properties of their leaf protein concentrates. Food Chemistry. 2002; 78: 63-68.

[5] Anderson JW, Smith BM, Gustafson NJ. Health benefits and practical aspects of high-fibre diets: Review Article. American Journal of Clinical Nutrition. 1994; 59: 1242S-1247S.

[6] Antia BS, Akpan EJ, Okon PA, Umoren IU. Nutritive and Anti-Nutritive Evaluation of Sweet Potatoes (*Ipomoea batatas*) Leaves. Pakistan Journal of Nutrition. 2006; 5(2): 166-168.

[7] AOAC. Official Methods of Analysis 14th ed. Association of Official Analytical Chemists. Washington DC, USA. 1984.

[8] Barnett T. HIV/AIDS, nutrition and food security: Looking to future challenges, pgs 341-348. In Gillespie S. 2006. AIDS, poverty and hunger: Challenges and responses. Proceedings of the International Conference on HIV/AIDS and food and nutrition Security, Durban, South Africa, April 14-16 2005. International Food Policy Research Institute, Washington DC. 2006.

[9] Brand JC, Cherikoff V, Lee A, Truswell AS. An outstanding food source of vitamin C. Lancet. 1982; 320(8303): 873.

[10] Bvenura C, Afolayan AJ. Analysis of heavy metal contamination in home gardens in Alice, Nkonkobe Municipality, Eastern Cape, South Africa. South African Journal of Science. 2012; 108(9/10): 1-6.

[11] Bvenura C, Afolayan AJ. Ethnobotanical survey of wild vegetables in Mbashe and Nkonkobe municipalities, Eastern Cape Province, South Africa. Acta Botanica Gallica: Botany Letters. 2014; 161(2): 189-199.

[12] Charles PC. Calcium absorption and calcium availability. Journal of Internal Medicine. 2009; 231(2): 161-168.

[13] Elbein RC. Nutrition and HIV infection. A continuum of care. Journal of the American Podiatric Medical Association. 1995; 85(2): 434-438.

[14] Faber M, Oelofse A, van Jaarsveld PJ, Wenhold FAM, van Rensburg JWS. 2010. African leafy vegetables consumed by households in the Limpopo and KwaZulu-Natal provinces in South Africa. South African Journal of Clinical Nutrition. 1995; 23(1): 30-38.

[15] Gupta S, Lakshimi JA, Prakash J. In vitro bioavailability of calcium and iron from selected green leafy vegetables. Journal of the Science of Food and Agriculture. 2006; 86(13): 2147-2152.

[16] Hart REC, Azubuike CU, Barmalaa IS, Achinewhu SC. Vegetable consumption pattern of households in selected areas of the Old Rivers State in Nigeria. African Journal of Food, Agriculture and Nutrition Development. 2005; 5(1): 1-19.

[17] Jimoh FO, Adedapo AA, Afolayan AJ. Comparison of nutritive value, antioxidant and antibacterial activities of *Sonchus asper* and *oleracea*. Records of Natural Products. 2011; 5(1): 29-42.

[18] Kawada T, Lee Y, Suzuki S, Rivai IF. Copper in carrots by soil type and area in Japan: A baseline study. Journal of Trace Elements in Medicine and Biology. 2002; 16(3): 179-182.

[19] Lewu FB, Mavengahama S. Wild vegetables in Northern Kwa-Zulu Natal, South Africa: Current status of production and research needs. Scientific Research Essays. 2010; 5(20): 3044-3048.

[20] Lopez MAA, Martos FC. Iron availability: An updated review. International Journal of Food Sciences and Nutrition. 2004; 55(8): 597-606.
[21] Lyimo M, Temu RPC, Mugula JK. Identification and nutrient composition of indigenous vegetables of Tanzania. Plant Foods for Human Nutrition. 2003; 58: 85-92.
[22] Medeiros DM, Wildman REC. Advanced human nutrition. 2nd Ed. Jones and Bartlet Learning, LLC. 2000.
[23] MINITAB. Version WINSV12.11. State College, PA: MINITAB Inc.; 1998.
[24] NHMRC. 2005. Nutrient Reference values for Australia and New Zealand: Including recommended dietary intakes. Canberra, Australia.
[25] NHMRC. Dietary guidelines for children and adolescents in Australia: Incorporating the infant feeding guidelines for health workers. Canberra, Australia. 2003.
[26] Nordeide MB, Hatloy A, Folling M, Lied E, Oshaug A. Nutrient composition and nutritional importance of green leaves and wild foods in an agricultural district, Koitiala, in southern Mali. International Journal of Food Science and Nutrition. 1996; 47: 455-478.
[27] Odhav B, Beekrum S, Akula U, Baijnath H. Preliminary assessment of nutritional value of traditional leafy vegetables in KwaZulu-Natal, South Africa. Journal of Food Composition and Analysis. 2007; 20: 430-435.
[28] Ogunlesi M, Okiei W, Azez H, Obakachi V, Osunsanmi M, Nkenchor G. 2010. Vitamin C contents of tropical vegetables and foods determined by Voltammetric and Titrimetric methods and their relevance to the medicinal uses of the plants. International Journal of Electrochemical Science. 2010; 5: 105-115.
[29] Schachter SM. The importance of magnesium to human nutrition. 2012. Available online: http://www.healthy.net/scr/article.aspx?Id=541 [Accessed on 26-01-2016].
[30] Shackleton CM, Pasquini M, Drescher AW. African indigenous vegetables in urban agriculture. Earthscan Publishers, UK and USA. 2009.
[31] Sharma N, Gupta PC, Rao CV. Nutrient content, mineral content and antioxidant activity of *Amaranthus viridis* and *Moringa oleifera* leaves. Research Journal of Medicinal Plants. 2012; 6(3): 253-259.
[32] Tiffin LO. Translocation of micronutrients in plants. In Micronutrients in agriculture, Proceedings of a symposium held at Muscle Shoals, Alabama USA, April 20-22, 1971; Mortveldvedt JJ, Giordano PM, Lindsay WL. (Eds).: Soil Science Society of America, Inc, Madison, Wisconsin, USA. 1971.
[33] Tinnerello D. HIV and nutrition. Body Posit. 1998; 11(9): 24-7, 30-1.
[34] UN/AIDS. 2010. Report on the global AIDS epidemic. 2010. Available online at: http://www.unaids.org/globalreport/documents/20101123_GlobalReport_full_en.pdf [Accessed 18-01-2016].
[35] Vijayakumari K, Siddhuraju P, Janardhanan K. Effect of soaking, cooking and autoclaving on phytic acid and oligosaccharide of the tribal pulse, *Mucunna monosperma* DC. Ex. Wight. Food Chemistry. 1996; 55(2): 173 – 177.
[36] Vorster IHJ, Venter LS, van Rensburg JWS. The importance of traditional leafy vegetables in South Africa. African Journal of Food, Agriculture and Nutrition Development. 2007; 7(4): 1-13.
[37] Wehmeyer AS, Rose EF. Important indigenous plants used in the Transkei as food supplements. Bothalia. 1983; 14(3/4): 613-615.
[38] Wheeler VE, Ferrel FE. A Method of Phytic Acid Determination in Wheat Fraction. Cereal Chemistry. 1971; 48: 312-316.
[39] WHO. No. 916. Technical Report Series: Diet, nutrition and the prevention of chronic diseases. Joint FAO/WHO expert consultation. 2003.
[40] Wilkinson SR, Welch RM, Maryland HF, Grunes DL. Magnesium in plants: Uptake, distribution, function and utilisation by man and animals. In: Sigel, H. –Sigel, A.: Metal ions in biological systems: Compendium on Magnesium and its Role in Biology, Nutrition and Physiology. Vol 26. Marcel Dekker, INC New York and Base. 1990.
[41] Yang Q, Liu T, Kuklina EV, Flanders WD, Hong Y, Gillespie C, Chang MH, Gwinn M, Dowling N, Khoury MJ, Hu FB. Sodium and potassium intake and mortality among US adults: prospective data from the Third National Health and Nutrition Examination Survey. Archives of Internal Medicine. 2011; 171(13): 1183-1191.

The black garlic

J. Sasaki

Department of Emergency Medical Technology, Hirosaki University of Health and Welfare Junior College, 2/5 Ohgimachi, Hirosaki 036/8104, Japan

Black garlic (*Allium sativum*) was a novel vegetable created by controlling humidity and temperature for a month without any additives. Under these conditions fresh garlic gradually changed color from white, brown and eventually became deep black at the end of processing. It is a chemical reaction called "*Mailard-Browning Reaction*". The final product is soft in texture, sweet with no pungent smell and directly edible like fruit. Water soluble amino acid *S-ally-L-cysteine* that is a trace in amount in fresh garlic increased by processing. Seventy percent amino acids (14/18) increased in amount along with elevation of carbohydrate contents. The black garlic extracts demonstrated a strong anti-tumor potency in a mouse tumor model accompanied with enforcement of NK cells activity. Bacteria-killing and anti-oxidant activities were also intensified by the black garlic extracts. Thus the multi-functional black garlic made sensationally debut armed with strong bio-functions in a field of vegetable world.

Keywords: black garlic (*Allium sativum*); *Mailard-Browning Reaction; S-ally-L-cysteine*; anti-oxidant potency; anti-tumor activity; anti-bacterial potency; immune enforcement

1. Introduction

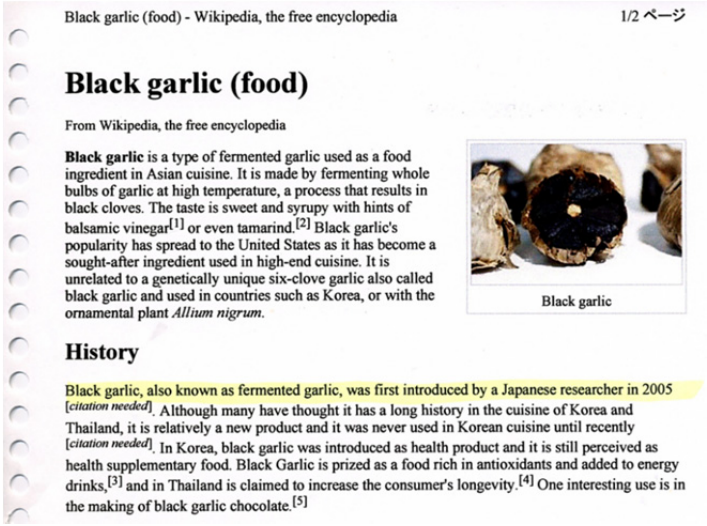
In the past garlic has been utilized as the most effective remedy against health disorders such as common cold, malaria, typhus, dysentery [1], and microbiologist *Louis Pasteur* scientifically studied the bactericidal properties of garlic. During the Second World War garlic was called "Russian penicillin" because the Russian government turned to this ancient treatment for soldiers when antibiotic supplies had been exhausted. More experimental data have been piling up against bacterial infection, atherosclerosis, high total cholesterol, hypertension, free radicals and blood coagulation [2]. Further the National Cancer Institute USA nominated garlic as the strongest cancer preventive vegetable on their "Designer Foods Project" [3].

In the vegetable world an extraordinary vegetable 'Black Garlic' made debut, which was developed by processing ordinary fresh garlic under the control of temperature and humidity [4]. The processed garlic was black in color with less irritating odor, fruity tasty, and readily edible without any further treatments like cooking. In this chapter, I will introduce extraordinary faces of the black garlic based on our experimental data.

2. History of the black garlic

On the history of the black garlic, people who read Wikipedia are understanding that it was created first in Korea. Whereas we Japanese know that the black garlic had been developed in our country. I will present herein a precise documents preserved in the Japanese company that was the first black garlic produced company. His name is Mr. *Hamasuke Hamano* who is still doing the black garlic business Mie Prefecture in Japan.

According to his documents he had initiated experiments in 1990 and succeeded in development of the black garlic in 1998. Wikipedia in the first edition had written the black garlic introduction following;



Black garlic (food) - Wikipedia, the free encyclopedia 1/2 ページ

Black garlic (food)

From Wikipedia, the free encyclopedia

Black garlic is a type of fermented garlic used as a food ingredient in Asian cuisine. It is made by fermenting whole bulbs of garlic at high temperature, a process that results in black cloves. The taste is sweet and syrupy with hints of balsamic vinegar[1] or even tamarind.[2] Black garlic's popularity has spread to the United States as it has become a sought-after ingredient used in high-end cuisine. It is unrelated to a genetically unique six-clove garlic also called black garlic and used in countries such as Korea, or with the ornamental plant *Allium nigrum*.

Black garlic

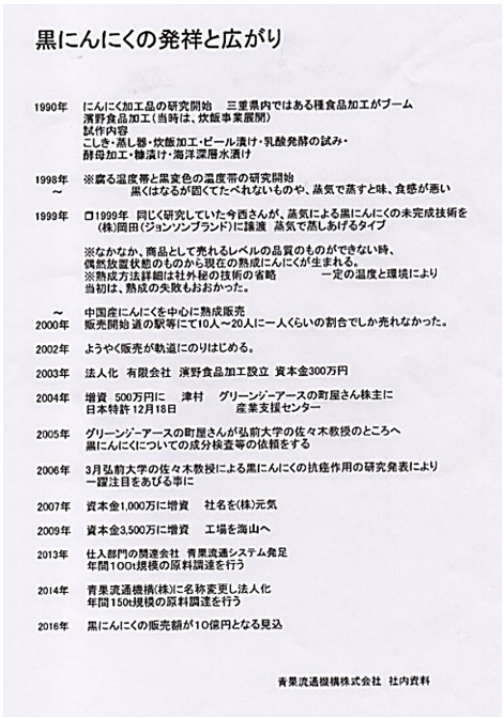
History

Black garlic, also known as fermented garlic, was first introduced by a Japanese researcher in 2005 [*citation needed*]. Although many have thought it has a long history in the cuisine of Korea and Thailand, it is relatively a new product and it was never used in Korean cuisine until recently [*citation needed*]. In Korea, black garlic was introduced as health product and it is still perceived as health supplementary food. Black Garlic is prized as a food rich in antioxidants and added to energy drinks,[3] and in Thailand is claimed to increase the consumer's longevity.[4] One interesting use is in the making of black garlic chocolate.[5]

Fig. 1 Black garlic descriptions in Wikipedia first edition. Pay attention to the first sentence of History saying Japanese researcher in 2005, it was my lab. work.

The above text said "It was first introduced by a Japanese researcher in 2005". It indicates our laboratory works. Later Wikipedia deleted this part to start from likely: "In Korea, black garlic was developed as a health product and it is still perceived as health supplementary food. Black garlic is prized as a food rich in antioxidants and added to energy drinks, and in Thailand is claimed to increase the consumer's longevity. It is also used to make black garlic chocolate."

This writing made people misunderstand the history of the black garlic. Here is the document by Mr. Hamasuke Hamano showing the date when the black was created in Japan (Fig. 2).


黒にんにくの発祥と広がり

1990年　にんにく加工品の研究開始　三重県内ではある種食品加工がブーム
浜野食品加工（当時は、炊飯事業展開）
試作内容
こしき・蒸し器・炊飯加工・ビール漬け・乳酸発酵の試み・
酵母加工・醤漬け・海洋深層水漬け

1998年 ～　※腐る温度帯と黒変色の温度帯の研究開始
黒くはなるが固くてたべれないものや、蒸気で蒸すと味、食感が悪い

1999年　□1999年 同じく研究していた今西さんが、蒸気による黒にんにくの未完成技術を
（株）岡田（ジョンソンブランド）に譲渡 蒸気で蒸しあげるタイプ

※なかなか、商品として売れるレベルの品質のものができない時、
偶然放置状態のものから現在の熟成にんにくが生まれる。
※熟成方法詳細は社外秘の技術の省略　一定の温度と環境により
当初は、熟成の失敗もおおかった。

～ 2000年　中国産にんにくを中心に熟成販売
販売開始 道の駅等にて10人～20人に一人くらいの割合でしか売れなかった。

2002年　ようやく販売が軌道にのりはじめる。

2003年　法人化 有限会社 浜野食品加工設立 資本金300万円

2004年　増資 500万円に　津村　グリーンジーアースの町屋さん株主に
日本特許 12月18日　産業支援センター

2005年　グリーンジーアースの町屋さんが弘前大学の佐々木教授のところへ
黒にんにくについての成分検査等の依頼をする

2006年　3月弘前大学の佐々木教授による黒にんにくの抗癌作用の研究発表により
一躍注目をあびる事に

2007年　資本金1,000万に増資　社名を㈱元気

2009年　資本金3,500万に増資　工場を海山へ

2013年　仕入部門の関連会社 青果流通システム発足
年間100t規模の原料調達を行う

2014年　青果流通機構㈱に名称変更し法人化
年間150t規模の原料調達を行う

2016年　黒にんにくの販売額が10億円となる見込

青果流通機構株式会社 社内資料

Fig. 2 Chronological description of the black garlic development (by the HAMANO Co. Ltd. document Mie Prefecture in Japan).

1990: The Hamano Food Processing Business initiated experiments to develop a direct edible garlic.

1998: Prototype of the black garlic was created but incomplete.

1999: The black garlic was developed using rice cooker. Mr. Hamano started a new business.

2003: Founded the Seino Food Processing Corporation.

2004: Acquired the Japanese Patent Right –Japan Patent Office– (December 18th).

2005: Bio-functional studies of the black garlic were carried out in our Laboratory at Hirosaki University School of Health Sciences.

2006: Research Group at Hirosaki University confirmed a strong anti-tumor potency, which was featured by the mass media. It became a trigger to establish the black garlic companies in Japan.

2007: The Hamano Company increased capital to 10 million yen and changed name to The Genki.

2014: The Vegetable & Fruit Distribution System was found.

According to Mr. Hamano's data, he was invited from Korea to give them technical advice for the black garlic production. They exported their products to California.

3. Experimental design

3.1 Creation of the black garlic

The black garlic is an ordinary garlic (*Allium sativum)* and not belongs to a special species. It is easily created under control of temperature (70ºC) and humidity (75%) for around 30 days without any additional treatments and additives. Fresh garlic gradually changes color from white, brown and eventually becomes black a month later as seen in Fig. 3 due to chemical reaction called "*Maillard and Browning Reactions*" between carbohydrate and amino acid. Final product is soft and sweet like fruit in taste with a non-irritating odor.

Fig. 3 Color changes during processing of ordinary garlic. Fresh garlic gradually changes color from white (left), light (5 days) and deep brown (10 days), and becomes deep black after 30 days treatment in the humidity and temperature controlled room.

3.2 Extraction of the black garlic compounds for bio-functions tests

The smashed black garlic was mildly heated in distilled water at 100ºC for 2 hours, and filtrated. Centrifuged supernatant (2,220×g for 20 min) was frozen at -80ºC overnight for lyophilization (BFD-2, Nihon Freezer Co. Ltd, Japan).

3.3 Chemical analysis

Amino acid, *S-allyl-L-cysteine* (SAC) and *γ-glutamyl-S-allyl-L-cysteine* (GSAC) were respectively analyzed by amino acid analyzer (JLC-500/v, Nihon-Denshi Co. Ltd, Japan), and liquid chromatography (Alliance 2696, Japan).

3.4 Anti-oxidant test

Anti-oxidant activity of the black garlic extracts was determined by the DPPH method, and expressed by mg used to reduce 50% of 1.1-diphenyl-2-picrylhydrazyl (RS50%).

3.5 Anti-tumor test

Meth A *fibrosarcoma* in BALB/c mouse model was used for anti-tumor test of the extracts. Briefly, tumor cells adjusted at 5.0×10^6/0.5 ml per mouse in GIT cell culture medium (Nihon-Seiyaku Co. Ltd, Tokyo, Japan) were transplanted into intra-dermal, and the extracts solution in disinfected saline was injected into tumor transplanted site on days 2, 4, 6 after transplantation. Anti-tumor potency of the black garlic extracts was evaluated three weeks later from tumor transplantation by measuring tumor size (longitude × latitude mm).

3.6 Toxicity test of the black garlic extracts against tumor cells

Toxicity of the black garlic extracts was tested against Meth A tumor cells at 5.0×10^7/ml suspended in 1% extracts contained-GIT medium. Followed by incubation in 5% CO_2-air, cell viability was microspcopically examined by trypan blue dye exclusion test at 3, 6, 9, and 24 hours cultivation interval.

3.7 Anti-bacterial test

Nutrient agar plate method [4] was adopted against the representative pathogens as MRSA (methicillin-resistant *Staphylococcus aureus*), enterohemorrhagic *Escherichia coli* O157:H7, *Pseudomonas aeruginosa* and *Candida albicans*. They are all troublesome organisms as drug resistance/food poisoning in the medical field and in the society.

3.8 NK (Natural Killer) cells activity test by spleen cells cultivation

Spleen cells culture system was adopted for NK cells activity test prepared from the black garlic extracts-fed mice [5]. Triplicate experiments were carried out for statistic evaluation.

3.9 Cytokine measurement in spleen cells cultivation system arranged by the extracts-fed mice

Cytokines amount of IL-2, TNF-α, IL-4, IFN-γ, and NO were measured in spleen cells culture supernatant after 48 hours incubation under 5% CO_2-air condition [5]. Triplicate experiments were conducted for statistic evaluation.

3.10 Statistic analysis

Chi-square (χ^2) was applied to evaluate the statistic significance between experimental and control groups.

4. Results

4.1 Chemical composition of the black garlic

Chemical constituents in the processed and fresh garlic were listed in Table 1. Carbohydrate in the black garlic increased in amount by processing, probably associated with enforced sweetness, but others did not change much in amount compared with those of fresh garlic.

Table 1 Chemical constituent of the black garlic and fresh garlic

	Black garlic	*Fresh garlic*
Energy (kcal/100 g)	227.1	138
Water (%)	45.1	60.3
Protein (%)	9.1	8.4
Lipid (%)	0.3	0.1
Carbohydrate (%)	47.0	28.7
Ash (%)	2.1	ND
Na (mg	4.0	ND
Ca (mg)	24.0	ND
Lactobacillus (No./g)	<300	ND

*ND; not determine

Carbohydrate amount rose by processing probable associated with black garlic sweetness.

Amino acid as cysteine, phenylalanine, tyrosine, leucine, isoleucine, methionine, valine, alanine, glycine, proline, glutamic acid, serine, threonine, and aspartic acid increased by processing (Table 2).

Table 2 Amino acid comparison between the black garlic and fresh garlic

Amino acid	*Black garlic (mg/100 g)*	*Fresh garlic (mg/100 g)*
Cysteine	260	100
Ricin	230	290
Histidine	110	130
Phenylalanine	300	190
Tyrosine	340	170
Leucine	460	260
Isoleucine	250	150
Methionine	90	70
Valine	410	250
Alanine	410	220
Glycine	360	180
Proline	210	180
Glutamic acid	1670	960
Serine	330	210
Threonine	270	190
Aspartic acid	930	630
Tryptophan	80	94
Arginine	970	1300

*Seventy percent of amino acid (14/18) increased during processing.

Organic-sulfur in the black garlic is considered to play a key role in bio-activity and *S-allyl-L-cysteine* (SAC) is one of the representative elements. SAC increased during processing, and its amount reached 194.3μg/g after 40 days processing to compare with 23.7μg/g before aging began. By contrast *γ-glutamyl-S-allyl-L-cysteine* (GSAC) was decreased from 748 to 248 μg/g after aging (Table 3).

Table 3 Relevancy between *S-allyl-L-cysteine* (SAC) and *γ-glutamyl-S-allyl-L-cysteine* (GSAC) in processing

	SAC (μg/g)	*GSAC (μg/g)*
Black garlic	194.3	248.7
Fresh garlic	23.7	748.7

SAC amount increased by processing but GSAC decreased, suggesting GSAC converted into SAC by processing.

4.2 Bio-activity potency of the black garlic

4.2.1 Anti-oxidant activity

Super-oxide elimination potency was strengthened in the black garlic and reached 28-fold more compared with that of fresh garlic in Japanese products and 12 times in Chinese products (Table 4), suggesting that activity depends on garlic producing area.

Table 4 Anti-oxidant potency of the black garlic (DPPH method).

	RS50%*
Japanese black garlic	4.1 – 28 times
Japanese fresh garlic	114.9
Chinese black garlic	7.3 – 12 times
Chinese fresh garlic	88.5

*mg used to reduce 50% of 1.1-diphenyl-2-picrylhydrazyl
Increasing anti-oxidant potency was observed in both Japanese and Chinese black garlic.

4.2.2 Anti-tumor activity

Tumor curative effect of the black garlic was more than expected. Fifty percent of tumor was deleted by the extracts treatments and fresh garlic used as a reference failed in eradication of tumor (Table 5). Average tumor size in non-cured mice in the black garlic treated was half to that of control group.

Table 5 Anti-tumor potency of the black garlic against Meth A *fibrosarcoma.*

	Dosage treated	***Cured/Used***	***Tumor size against control (%)***
Exp.1 Black garlic	1 mg (three shots)	2/5	40
Control	(-)	0/5	
Exp.2 Black garlic	1 mg (three shots)	3/5	55
Control	(-)	0/5	
Total		5/10 ($p<0.05$)	47.5
Fresh garlic	5 mg (three shots)	0/5	64
Control	(-)	0/5	

Extracts were injected into tumor transplanted site on day 2, 4, 6 after tumor transplantation, and activity was evaluated three weeks later from tumor transplantation.
Fifty percent of cure rate was obtained by the black garlic treatment, and no cure in the fresh garlic treated group.

4.2.3 Toxicity test of the black garlic extracts against Meth A tumor cells

One percent of the black garlic extracts was no toxic against Meth A tumor cells in mixture cultivation test (Fig. 4). This result suggests that the extracts probably enhance immune system first, which will play later for tumor eradication *in vivo*.

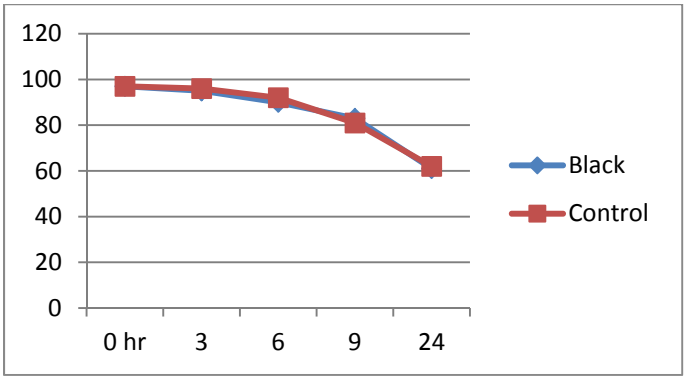


Fig. 4 Toxicity test of the black garlic extracts against Meth A tumor cells.

One percent of the black garlic extracts was mixed with tumor cells and incubated in 5% CO_2-air condition. Viability rate of tumor cells (%) was determined by the dye exclusion test counting 200 cells. Non toxic action was observed in the black garlic extracts.

4.2.4 Anti-bacterial test

MRSA (methicillin-resistant *Staphylococcus aureus*), *Pseudomonas aeruginosa,* and enterohemorrhagic *Escherichia coli* O157:H7 were sensitive to the extracts, and could not grow on the extracts included site. While *Candida albicans* resisted somewhat to the extracts and faintly grew as seen in Fig. 5.

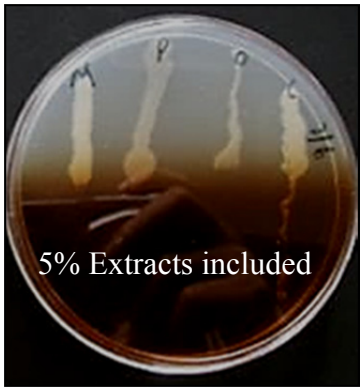


Fig. 5 Bacteria killing potency of the black garlic extracts. Upper control site allowed bacteria growth but no growth at lower part with the black garlic extracts. From left; MRSA, *Pseudomonas*, O157, *Candida* (faint growth)

4.2.4 Enhancement of NK cells activity

As seen in Fig. 6, NK cells were activated and it reached maximum 10 days after the experiments beginning (p<0.001).

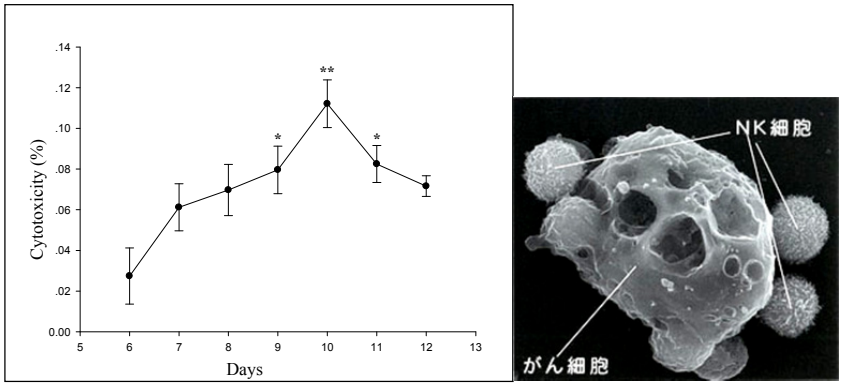


Fig. 6 Enforcement of NK cells activity in the black garlic extracts-fed mice. NK cells activity gradually increased and reached a maximum on day 10 (*P<0.05, **P<0.001 against control value). Tumor cell was attacked by NK cells by creating holes (Right picture, by Hasumi Institite in USA).

4.2.5 Increased cytokine generation

Cytokine TNF-α, and IFN-γ reached a maximum generation on day 8, and IL-2 and NO were on day 11 (Table 6). Time lag was observed on reaching maximum depending on the types of cytokine. While IL-4 decreased, implying less production of *IgE* from B lymphocytes (allergy suppression).

Table 6 Cytokine amount generated in the extracts-fed mice spleen cells culture.

Days	***IL-2 (pg/ml)***	***IL-4 (〃)***	***TNF-α (〃)***	***IFN-γ (〃)***	***NO (μM)***
Control	20.1± 3.9	20.0±2.1	27.3±8.2	17.4±1.5	11.6±0.9
6	15.9 ± 2.3	16.6±2.8	47.0±5.9*	24.5±1.7*	11.2±2.3
7	15.5±1.8*	17.7±1.7	60.7±3.9*	34.7±4.3**	10.4±1.2*
8	18.2±4.0	15.5±1.0*	68.7±0.6**	105±55.7*	26.9±1.0**
9	18.0±2.6	15.9±1.3*	56.5±3.4**	30.0±4.9*	26.1±1.1**
10	16.2±1.8	15.9±1.6*	51.0±8.7*	18.1±0.7	26.3±1.8**
11	28.0±3.6*	16.5±1.0*	44.0±5.4*	32.2±4.3*	28.8±2.9**
12	18.6±3.0	17.2±1.2*	29.0±5.4	19.7±1.4	26.0±2.5*

Mice were treated with the black garlic extracts for 5 days. Non-treatment period was from day 6 to 12. (*P<0.05, **P<0.001 against control).

5. Prospect in the future

Reputation of the novel vegetable, black garlic, is steadily raising up in both domestic and overseas due to beneficial bio-functions against tumor, super-oxide, bacteria, and immune enhancement. Citizens are also interested in their health problems and trying to strengthen their health conditions. The most hopeful candidate is the black garlic, which still covered with unknown nature (bio-activities).

One of the biggest concerns is for instance how this product works against a modern disease allergy. We are experiencing the black garlic effectiveness against allergy as improvement of clinical symptoms among the patients who daily ingested the black garlic. Another concern is affection to the brain. Because the black garlic involves much γ-*amino-butyric acid* (GABA), which is a necessary element to brain work as a neurotransmitter in central nervous system [6]. Deficiency of GABA in brain causes various mental diseases as anxiety disorders likely to be panic attacks, Parkinson disease, and also depression. Likely the black garlic bio-functions are still unexplored and remained for future's works.

Acknowledgments Author acknowledges Mr H. Hamano and Y. Osanai for providing information on the history of the black garlic in Japan.

References

[1] Bratman S. Your Complete Guide to Herbs. Prima Publishing. California, pp 67-75, 2000
[2] Sasaki J. Bioactive Phytocompounds and Products Traditionally Used in Japan. In Modern Phytomedicine -Turning Medical Plants into Drug- Edited by Iqbal Ahmad et al. WILEY-VCH. pp79-96, 2006
[3] Articles.latimes.com/1991-12.../food/fo-1157_1_cancer-research
[4] Sasaki J, Lu C, Machiya E, Tanahashi M, Hamada K. Processed black garlic (*Allium sativum*) extracts enhance anti-tumor potency against mouse tumors. Medicinal and Aromatic Plant Science and Biotechnology. Global Science Books. 1 (2), 278-281, 2007
[5] Wang D. Feng Y. Liu J. Yan J. Wang M. Sasaki J. Lu C. Black garlic (*Allium sativum*) extracts enhance the immune system. Medicinal and Aromatic Plant Science and Biotechnology. Global Science Books. 4 (1), 37-40, 2011
[6] Sasaki J. Overview of the Black Garlic Movement in the Fields of Research and Marketing. J Life Sciences. 9 (2), 65-74, 2015

The environmental hazards of food wastage: A brief summary

M. Asaduddin Laskar[1,*], **S. Kashif Ali**[1] and **Sana Siddiqui**[2]

[1] Department of Chemistry, Faculty of Science, Jazan University, Jazan, Saudi Arabia
[2] Department of Chemistry, College of Science and Arts, Samtah, Jazan University, Saudi Arabia

The increasing volume of wastage of food has become a global problem. According to a recent report of Food and Agriculture Organization of the United Nations, food waste causes economic and ecological deterioration. This chapter would discuss the different food supply chains and occurrences of food wastage. The appropriate indicators for environmental deterioration of food waste, namely acidification, eutrophication, global warming, human and eco-toxicity and depletion of resources, would be discussed. The chapter would also focus on the potential measures for the prevention of food wastage. It would then conclude the critical factors at the end.

Keywords: Food wastage; environmental impact; indicators

1. Introduction

Food wastage refers to the reduction in the availability of food for consumption occurring throughout the food chain, both due to lack of efficiency (poor infrastructure, poor technology and poor management) and due to unintentional discarding of edible items at the retailer's and consumer's end [1]. Globally, food waste is being generated in enormous amount and has become a major cause of concern because of its impact on the environment. About 50 % of the food is wasted globally through the food supply chain [2]. The food waste has not been measured or managed in a regular manner. As for instance, food and organic wastes are often considered together, whereby it becomes difficult to figure out or measure the food-only portion, while in other cases, the reporting is considered for mixed solid waste, only at the stage of disposal [3].

1.1 Food crises

The price of food has been low till the early 2000s, and therefore, the governments of both developing and underdeveloped countries did not invest much in the local agricultural production. However, the reliability on food imports, for food security, suffered a setback when the international food prices started soaring since 2006. As an immediate reaction, such surge in food prices leads to widespread riots and protests in many countries which threatened the stability of the respective governments [4]. According to the report of World Bank, in 2011, about 44 million people were pushed into poverty, as the food prices reached the unaffordable limit [5]. Such situation where a substantial population is forced to starve for food is generally referred to as the "food crisis". It is estimated that about 925 million people are subjected to chronic hunger, throughout the world [6]. However, besides food production, a sustainable food system may also depend on factors including food consumption and food wastage.

1.2 Food supply chains

The food supply chain is a combination of various stages (interlinked to each other) through which food reaches the consumer. These stages may be broadly categorized into two groups, namely inbound logistics and outbound logistics. The inbound logistics may include facilities such as suppliers (delivery of raw materials), factories (manufacturing and packaging), while the outbound logistics may include transportation to storage facilities that is accessible to the consumers (distribution centers and retail outlets). Again, the supply chains, for different products, may intersect at one or more stages. [7].

This chapter would therefore discus in length the occurrences, indicators and prevention of food wastage.

2. Occurrence of food wastage

According to the report of Food and Agricultural Organization of the United Nations (FAO), about one-third of the food produced worldwide, amounting to 1.3 billion tons (per year), is wasted [8].

The wastage of food may take place through spillage, breakage and degradation (especially, during handling and transportation). The environmental impact becomes more severe/or intense with the passing stages (along the food supply chain), as the impacts (or cost) is added to the initial production impact. The wastage may occur at any part of a supply chain: production (at agricultural production), manufacturing and processing, wholesaling and retailing, as well as at the consumer's end. The loss of raw, intermediate or final products may also occur during international and/or domestic trading. The different parts of the food supply chains, of vegetable and animal commodities, where the probable food waste occurs may be depicted as follows [9]:

2.1 Production stage

During harvesting of the agricultural products, their accidental or unavoidable damage and/or spillage may occur. Again, during the handling, storage and transportation of the harvested crops, a portion may get spilled or damaged (through degradation). During breeding, animals and/or birds may die due to sickness and/or accidents. Again, during fishing there is always a discarded portion, while at dairy production units, the cows may get sick whereby its production of milk get reduced. Animals may die during transportation and fishes are lost during icing, packaging, storage and transportation. The produced milk may get spilled or degraded on its way to the subsequent unit.

2.2 Manufacturing and processing

Besides the losses due to spillage (or degradation) at the manufacturing units, a significant portion may also get lost at preparation units, such as washing, slicing etc., prior to their introduction to the former. At the industrial processing unit, meats are lost due to trimming spillage and fishes are lost during canning and smoking. Again, during the treatment of milk, such as pasteurization or processing of cheese/yoghurt, a substantial portion gets spilled.

2.3 Wholesaling and retailing

Losses may take place during handling, shifting, storage and transportation at the market system.

2.4 At consumer end

The wastage of food at the domestic level, namely handling, preparation and consumption.

3. Indicators for environmental deterioration by food wastage

LCA is a process of evaluating the effects that a product has on the environment over the entire period of its life.The LCA study starts with the identification of objectives. The product system is then examined in terms the impact categories namely acidification, eutrophication, global warming, eco-toxicity (in relation to human) and depletion of resources. The technique of Life Cycle Assessment (LCA) is one of the effective tools for studying the environmental impacts, as it considers all the stages of a food supply chain. This tool facilitates the investigation and evaluation of the effects (on air, water and land) of stages (of food supply chain), namely agricultural production, material and component preparation, distribution, consumption and end-of-life management (such as disposal, recycling etc). Certain preselected reference substances have been used to express the results of LCA, like CO_2 for climate change impact, SO_2 for acidification and ethene (C_2H_4) for Photochemical Ozone creation potential etc [10].

3.1 Acidification and Eutrophication

Acidification refers to the emission of gasses, such as SO_2, NOx, HCl and NH_3, into the air, which subsequently interact with the atmosphere to acidify the ecosystems. *Eutrophication* is the excessive growth of plants, namely algae, periphytons and weeds, within water bodies (such as lakes, streams and estuaries), caused by the excessive nutrients content produced as a result of the presence of gasses, namely NOx, NH_3, PO_4^-, in water and air. The sources of such nutrients may include fertilizers (from agricultural fields), soil erosion, discharge from sewage treatment plants, atmospheric nitrogen and run-off from lawns/fields. Eutrophication may cause the reduction of oxygen, during the decomposition of dead plants, which would be fatal for other organisms. Pollution of water resources with pesticides and fertilizers may stimulate the excessive growth of aquatic plants and algae, which would result in clogged waterways, depletion of dissolved oxygen and blocks sunlight from reaching deeper. It has detrimental effect on the process of respiration of aquatic life [11].

Nitrate is leached from all types of soils and gets carried away by the drainage water. The sewage treatment plants also discharges substantial amount of nitrate. These sources of nitrate contribute to the eutrophication of water bodies, mainly, coastal waters, marine waters and lakes. [12]. The soil, in the agricultural land, is contaminated with nitrate during the decomposition of organic matter (including manures). This nitrate is transported to the drainage water during rainfall. Again, excess of phosphorus gets deposited into the soil (of farmland) due to the practice of adding inorganic phosphate fertilizers. The excreta of the animals (including human sewage), which are fed with the crops grown on phosphate contaminated soil, also contain phosphorus. Thus, the phosphorus gets carried away into the waterways. Ammonia is released into the soil through livestock wastes (particularly from cattle, poultry and pigs). The release of ammonia into the air leads to the formation of ammonium particles, which causes smog formation as well as acidification. [12].

3.2 Global warming

Global warming indicates the trapping of a portion of the reflected outgoing solar energy, whereby retaining heat into the lower atmosphere in the vicinity of the earth's surface. Such phenomenon is caused by the emissions of greenhouse gasses (namely, CH_4, N_2O and CO_2) into the atmosphere.

The soil releases CO_2 through its respiring plant roots as well as decomposed organic matter (caused by soil microbes). The N_2O gas is released from the soil due to the processes of nitrification and denitrification (carried out by microorganisms), which are initiated in the presence of nitrogen (in the soil). The soil is being continuously fed with nitrogenous compounds in the form of fertilizers, feces, slurries, manure etc. The CH_4 gas is being released from anaerobic soils and animal wastes. Food wastage of cereals produces 34 percent of the total greenhouse gases [13]. The main causes are the application of nitrogenous fertilizers (during production) and usage of diesel (during agricultural operations, namely ploughing, harvesting and drying). Such usage of nitrogenous fertilizers reduces during the production of pulses as the latter, being a leguminous plant, has the ability to fix nitrogen from air. Similarly, the requirement for nitrogenous fertilizers and diesel is less for the production of potatoes and other roots, thanks to their efficient and high yield. However, the production of grains requires higher usage of nitrogenous fertilizers and diesel as compared to fruits production. The monogastric animals, such as pigs and poultry, contribute to N_2O emissions through their feeder, while CO_2 emissions occur during the production of mineral fertilizers. The usage of energy during maintenance of the animal's shelters also adds to the overall emissions due to certain animals (like chicken). The ruminants, including cattle, sheep and goats, contribute to the emissions of CH_4 gases, especially during feed digestion (due to enteric fermentation) and manure management. Additionally, N_2O emissions also occur during the production of fertilizers, soil emissions and energy usage (in arable farming). In fisheries, the consumption of diesel (during on-board fishing) and leakage of refrigerants (from on-board cooling equipment) adds to the CO_2 emissions. In aquaculture, the emission of greenhouse gases occur during the production of fishmeal and fish oil (in industrial scale) [14].

3.3 Human and eco-toxicity

Human and eco-toxicity refers to the exposure to pesticides and heavy metals which cause harm to human and the ecosystem (consisting the flora and fauna). The rising occurrence of pest and disease has prompted the use of enormous amount of hazardous pesticides [15]. Generally, herbicides are less hazardous to human health than insecticides [16]. In agriculture, environmental hazards include the emissions of gases (such as ammonia), pollution of surface and groundwater due to nitrate and/or phosphate leaching, erosion of soil and harmful effect on biodiversity [17,18].The excessive use of fertilizers and pesticides, in modern agriculture, has negative impact on the natural habitats as well as genetic diversity. As for instance, a sensitive plant like tobacco needs multiple applications of pesticides, fungicides and herbicides during its growing season [19]. Every year 27 million pounds of pesticides are sprayed onto tobacco fields in the United States [20]. Besides causing harm to birds and small animals, it causes "green tobacco sickness", which affects the field workers who are constantly exposed to these pesticides [21]. Besides carbon dioxide, enormous amount of solid waste and/or chemical waste (including paper, ink, foil, glue and cellophane) are generated in the production of cigarettes [22]. Moreover, tobacco smoke is known to contain toxic substances (including carcinogens) [23]. About 0.1% of the applied pesticides reach its target, while the remaining is released into the environment and thus contaminates soils, plants, air and water bodies. The effects of pesticides on living species lead to tropic effects on biodiversity. The application of sewage sludge on land introduces a wide range of toxic heavy metals and organic compounds which subsequently gets assimilated by plants and animals [12].

3.4 Uses of resources

Use of resources indicates the depletion of resources (especially non-renewable), namely fuels, water and agricultural land, due to extraction and consumption. An instance of the depletion of resources includes the use of cropland and/or grassland for the production of that portion of the total foodstuffs which is ultimately wasted. Similarly, during the energy-intensive processing of food, solid waste and wastewater is generated. During the preparation of food at home, substantial amount of energy is used for freezers, refrigerators and cooking and thus has a negative environmental impact.

Land may be used for agriculture, buildings or roads , which may result in deforestation, urbanization and soil sealing. Again, aquaculture may be interconnected to land-use by virtue of the agricultural products used as feed for fish. During livestock production, lands are occupied for the production of animal feed including grazing. As for instance, ruminants are fed with roughages and/or grains and soy-meal as well as other supplements. Again, monogastric animals may also be connected to non-arable land as their feed may contain components of milk from ruminants that require grassland. Among food crops, cereals contribute about 4-15 % of the total arable land occupation of food wastage. However, starchy roots, vegetables and legumes compensate for their food wastage volumes with high yields. At the agricultural production stage, 99% and 50% of food wastages occur in the regions with moderately (and/or highly) and lowly degraded soil, respectively.

The biodiversity comprises of life on earth including diverse genes, species and ecosystem. The greatest harm to tropical biodiversity may be caused by the extension of crop production. The industrial and urban expansions,

especially in the developed countries, have caused the loss of habitat, pollution of the ecosystem and the reduction in the farmland diversity. Moreover, the growing abandonment of agricultural land has led to the decline in the habitat heterogeneity, whereby causing a further decline in biodiversity. In animal husbandry, large stretch of natural areas are converted to pastures as well as used for forage production, which in turn has enormous impact on the biodiversity. Additionally, decline in the genetic diversity of livestock also affects the biodiversity. Biodiversity is also influenced by the alteration of the marine ecosystem prompted by the increasing fish and/or seafood production. The modern industrial fishing destroys sea-floor habitats. Moreover, the discarded alien/unwanted species (as waste), the continuous introduction of hormones and/or antibiotics, the genetic mutation (of wild fish) and the transmission of diseases have narrowed the diversity of the biosphere.

Land with thin topsoil layer suffers from soil loss due to conventional tillage and heavy rainfall [12]. About 60% of used water resources goes to agriculture [24]. In horticulture, the practices of using glasshouses, poly-tunnels and mulches (and/or fleeces) affect the landscape [25]. The depletion of water resources (suitable for use) is caused by its pollution through the improper use of pesticides and fertilizers.

4. Measures for prevention of food wastage

Ecological agriculture (or agro-ecology) involves intercropping, recycling of manure and food scraps into fertilizers, agro-forestry (to maximize resource efficiency) and many other agronomic techniques. Variation in farming systems leads to diverse diets and thus improves the nutritional level. It reduces the expenses (for farming) and thus contributes to better livelihoods. Shifting the mode of agriculture from industrial to ecological could be easily done (both national and international) with the cooperation of the respective government agencies [26]. Introduction of food info-Marts (market for ecological product) is an efficient and effective tool through which the flow of information (and resources) could be mutually shared and/or exchanged.

The development of Ecological Debt Index which would take into account the overall carbon emissions, water consumption, application of chemical fertilizers and/or pesticides on agricultural land etc, would indicate the ecological impact of the product (throughout the food supply chain). A food label, indicating the ecological debt index, would be introduced for all product so that the consumers can make their choice/or preference for product received through greener supply chains.

Reduction of food wastage may be accomplished by improving poor (or inappropriate) practices along the different stages (of food supply chain), namely agricultural, processing, transportation and distribution . Methods like by-product recycling, anaerobic digestion, composting and incineration are advantageous (and beneficial) over mere dumping in landfills [27].

Fertilizers contribute to the emission of green house gases by virtue of the inefficient manufacturing technologies, use of coal (as energy source), preference of urea fertilizers over ammonium nitrate, and its over-application (especially in horticulture) [28, 29]. Improved fertilizer management, conservation tillage, use of biogas as well as farming of productive livestock breeds by using feed additives and antibiotics are some important adoptive measures to reduce emissions. In organic farming, diversified crop rotation is practiced and the use of pesticides, herbicides or inorganic fertilizers is avoided. It enhances biodiversity (in agricultural landscapes), increases species richness by 30% [30]. The practice of high yielding crop production with efficient nitrogen use may diminish the need for synthetic fertilizers, expansion of agricultural land and change of forests (habitats) of technologies, namely slurry injection, manure treatment and well-timed manure application, serve the purpose of maintaining ammonia reductions, nitrogen conservation and reduced fertilizer application. The adoption of anaerobic digestion method promises to improve nitrogen efficiency by replacing synthetic fertilizers with the nitrogenous by-product of anaerobic digestion. The practice of intensified agriculture on suitable productive soils may encourage the slowing and reversing of deforestation [31]. Use of buffer strips and spatial planning for livestock production reduces emissions of green house gases [32].

The practice of sustainable agriculture refers to the use of resources without compromising with the environmental quality and quantity of natural resources. Agricultural sustainability demands optimal utilization of water, crops, soil fertility and soil physical properties. Such practice minimizes chemical and energy inputs and helps improve soil fertility and crop production through synergic ecological interactions [11].

5. Conclusion

Although biodiversity and the effects of varying agricultural practices on landscape do not fall within the domain of LCA methods, however, it covers several important factors, including greenhouse gas emissions (affecting climate), acidifications (acid gas emissions), eutrophication (nitrifying emissions), ozone depleting substances and depletion of resources (both biotic and abiotic). LCA takes into consideration the impacts on all media of the environment, namely land, air and water [10].

Deforestation causes an increase in the atmospheric level of carbon dioxide, severe flooding, soil degradation and restricts water recycling (of nature), which ultimately leads to the extinction of plant and animal species [33,34]. The

treatment of waste, generated by livestock farms, is normally inappropriate, as reported in the state of the environment report 2002 [35]. Livestock waste contributes substantially to the release of COD (chemical oxygen demand), Nitrogen, Phosphorus and heavy metals [36]. The released nitrogen enters the atmosphere in the form of N_2O and/or NH_3, accumulates into soils and contaminates water bodies. The food supply chain may affect the nutritional quality of food. As for instance, fresh vegetables undergo deterioration during transportation, handling and storage [25]. Adoption of preservation methods, namely refrigeration, chlorination, electrolyzed water treatment, ionization (through radiation), packaging and surface coating, may increase the shelf-life of fresh vegetables as well as reduce nutritional loss and physical damage [37].

Water is inseparable in every aspects of life and hence special attention should be given to the development, utilization and protection of water in both the developed and developing countries. The inappropriate use of water for irrigation may cause water depletion, salinization, water –logging or soil degradation.

Adoption of strategies (in sustainable agriculture), namely tillage, crop rotations and use of plant residues as manure, affects soil habitats and the food web and also changes the quality of the soil. The soil biota is involved in the process of decomposition (of organic matter) and maintains the nutrient and carbon cycles. Plants play a crucial role in maintaining the porosity and organic matter content of the soil. Plants are also involved in the bioremediation of toxic wastes [38].

References

[1] Steenbergen H. Is Cheese Sustainable? A Multi-scale Exploration of Botton Gouda; 2013. Available from:https://ojs.st-andrews.ac.uk/index.php/jsus/article/download/606/526

[2] Smil V. Feeding the World: A Challenge for the 21st Century. Cambridge: The MIT Press; 2000.

[3] Mason L, Boyle T, Fyfe J, Smith T, Cordell D. National Food Waste Data Assessment: Final Report. Prepared for the Department of Sustainability, Environment, Water, Population and Communities, by the Institute for Sustainable Futures, University of Technology, Sydney: Sydney; 2011.

[4] Rising Food and Energy Prices in Europe and Central Asia. Report No. 61097. Washington DC: The world bank; 2011. Available from: http://www.worldbank.org/en/region/eca/publication/rising-food-and-energy-prices-in-europe-and-central-asia

[5] The global food crises. United Nation; 2011. Available from: http://www.un.org/esa/socdev/rwss/docs/2011 /chapter4.pdf

[6] Sustainable Development Goals, Food and Agriculture Organization of the United Nations. Available from: http://www.fao.org/sustainable-development-goals/en/

[7] Wakeland W, Cholette S, Venkat K. Food transportation issues and reducing carbon footprint. In: Boye JI, Arcand Y, editors. Green Technologies in Food Production and Processing. Food Engineering Series, LLC: Springer; 2012.

[8] The State of Food insecurity in the world 2014. FAO, World food Program; 2012.

[9] Global food losses and food waste – Extent, causes and prevention. Rome: FAO; 2011.

[10] Foster C, Green K, Bleda M, Dewick P, Evans B, Flynn A, Mylan, J. Environmental impacts of food production and consumption: A report to the department for Environment, Food and Rural Affairs. Defra, London: Manchester Business School; 2006.

[11] Krishnan P. Future challenges of providing high-quality water-vol. II- Environmental Impact of Food Production and Consumption, Encyclopedia of Life Support Systems (EOLSS). Available from: http://www.eolss.net/Eolss-sampleAllChapter.aspx.

[12] Murphy-Bokern D. Environmental impacts of the UK food economy with particular reference to WWF Priority Places and the North-east Atlantic; 2008.

[13] GHG data from UNFCCC. Available from: http://unfccc.int

[14] Food wastage footprint: Impacts on natural resources. FAO. Available from: www.fao.org/docrep/018/i3347e/i3347e.pdf.

[15] BCPC. In: Whitehead R, editor, The UK pesticide guide (2007).Wallingford, UK: BCPC/CABI; 2007.

[16] Cross P, Edwards-Jones G. Variation in pesticide hazard from vegetable production in Great Britain from 1991 to 2003. Pest Management Science. 2006; 62: 1058-64.

[17] Havlikova M, Kroeze C. Evaluation of methods for quantifying agricultural emissions of air, water and soil pollutants. Science of the Total Environment. 2006; 372: 133-47.

[18] Almasri MN, Kaluarachch JJ. Modelling nitrate contamination of groundwater in agricultural watersheds. Journal of Hydrology2007; 343: 211-29.

[19] Action on Smoking and Health (ASH). (2009) ASH fact sheet: Tobacco and the environment. Available from: http://www.ash.org.uk/files/documents/ASH_127.pdf

[20] McDonough S. Potent pesticides used on tobacco. 2009, Feb 11. Available from: http://www.cbsnews.com/2100-205_162-550925.html

[21] Fact Sheet: Environmental Impact of Tobacco. Multnomah Tobacco Prevention and Education Program; 503-988-4163; 2013. Available from: htlh.tobacco.prevention@multco.us

[22] McLaren W. Smoking: Environmental and social impacts. 2007, Feb 27. Available from: http://www.treehugger.com/green-food/smoking-environmental-and-social-impacts.html

[23] Repace JL Fact sheet: Outdoor pollution from secondhand smoke. Available from: http://www.repace.com/pdf/OTS_FACT_SHEET.pdf

[24] Ministry of Water Resources. Water Resources Bulletin. Beijing; 2011.

[25] Edwards-Jones G, Canals LM, Hounsome N, Truninger M, Koerber G, Hounsome B, Cross P, York EH, Hospido A, Plassmann K, Harris IM, Edwards RT, Day GAS, Tomos AD, Cowell SJ, Jones D L. Testing the assertion that 'local food is best': the challenges of an evidence-based approach. Trends in Food Science & Technology. 2008; 19: 265-74.
[26] An IGES-CSA venture 2016-17. Strengthening Food Resource and Information Channels for Sustainable Production and Consumption of Food. Natural Resources and Ecosystem Services Area Institute for Global Environmental Strategies. Hayama, Japan. Available from: https://pub.iges.or.jp/pub_file/brochurelow/download?token=Cbuo75-g
[27] Food waste harms climate, water, land and biodiversity – new FAO report. Rome; 2017. Available from: www.fao.org/news/story/en/item/196220/icode/
[28] SAIN. Policies and technologies to overcome excessive and inefficient use of nitrogen fertiliser: delivering multiple benefits. SAIN policy brief no. 5. Norwich: Sustainable Agriculture Innovation Network; 2012.
[29] Xin L, Li X, Tan M. Temporal and regional variations of China's fertiliser consumption by crops during 1998–2008. Journal of Geographical Sciences. 2012; 4: 643-52.
[30] Bengtsson J, Ahnstrom J, Weibull AC, The effects of organic agriculture on biodiversity and abundance: a meta-analysis. Journal of Applied Ecology. 2005; 42:261-69.
[31] Sommer R., Vlek, PLG, Rodriguez-Kuhl, G. Energy use and CO_2 production in tropical agriculture and means and strategies for reduction or mitigation. Environment, Development and Sustainability. 2004; 6: 213-33.
[32] Forti R, Henrard M. Agriculture, forestry and fishery statistics. Luxembourg: Eurostat; 2016. Available from: http://ec.europa.eu/eurostat.
[33] Matthews C. Deforestation causes global warming. 2006, Sept. 4. Available from: http://www.fao.org/newsroom/en/news/2006/1000385/index.html
[34] Hilderman R. The effect of deforestation on the climate and environment. 2010, Dec. 27. Available from: http://www.motherearthnews.com/understanding-climate-change/the-effect-of-deforestation-onthe-climate-and-environment.aspx
[35] State of the Environment Report 2002. Beijing: State Environment Protection Agency; 2002.
[36] First National Pollution Sources Census. Beijing: Ministry of Environmental Protection; 2010.
[37] Alzamora SM, Tapia MS, Lopez-Malo A, editors. Minimally processed fruits and vegetables: Fundamental aspects and applications. Gaithersburg, MD, USA: Aspen Publishers, Inc; 2000.
[38] Food wastage footprint: Impacts on natural resources: summary report. Natural Resources Management and Environment Department; 2013. Available from:www.fao.org/publications

The Functional Aspects of Beta Glucan for Dairy Industry

Tulay Ozcan*, **Arzu Akpinar-Bayizit** and **Lutfiye Yilmaz-Ersan**

Department of Food Engineering, Uludag University, 16059 Gorukle, Bursa, Turkey
* Corresponding author: email: tulayozcan@uludag.edu.tr

Beta glucan (*β*-glucan), an important functional ingredient, has gained renewed interest from the food industry due to its health beneficiary compounds beyond basic nutrition. This soluble fiber, with high water-holding capacity and gelling, thickening, stabilizing and emulsification properties, is readily found in the cell walls of cereal grains (i.e. barley and oats), algae, bacteria and fungi. In scientific literature, beta-glucan has been documented for playing beneficiary role in insuline resistance, hypertension, and obesity; reduction of serum cholesterol levels; helping the production of short chain fatty acids and forming highly viscous solutions that promote the growth of beneficial gut microflora, and consequently, lowering the risk of cancer. In dairy products *β*-glucan is being widely used either to improve microstructure and rheological properties or enhance their health properties such as calorie-reduction and cholesterol-lowering. The objective of the present paper is to explore the role of *β*-glucans as a source of dietary fiber supplementation, their potential uses in dairy applications and development of new nutraceutical products.

Keywords: health benefits; *beta*-glucan; fiber; functional dairy foods

1. Introduction

Food industry aims to develop functional foods and ingredients with regard to the consumers' attitudes and behavior towards healthy and nutritous foods. *β*-glucans are commonly referred as dietary fibre. A dietary fibre, non-starchy carbohydrates, can be defined as the parts of plants such as cereals, vegetables, fruits, and nuts which are not digested within the small intestine since mammals do not produce enzymes capable to hydrolyze them into constituent monomers [1].

Based on their simulated intestinal solubility, dietary fibres are classified as either soluble or insoluble. Soluble fibres include pectins, beta-glucans, galactomanan gums, and a large range of non-digestible oligosaccharides i.e. inulin; insoluble fibres include lignin, cellulose, and hemicelluloses [2]. Dietary fibre is a non-nutrient as it reaches the colon intact and contributes no calories to the diet. In the colon, it is fermented by the beneficiary bacteria, and metabolized to short chain fatty acids (SCFAs) with the release of energy that promotes the growth of gut microbiota, acting as prebiotic [3-5].

2. Sources of *β*-glucans

2.1 Structure and functional properties of *β*-glucans

Glucans are non-starch polysaccharides commonly present in living organisms. They are polymers built of monosaccharides linked by alfa- and beta-type glycosidic bonds. Glucans have complex chemical structure and perform varied physicochemical properties. They can be divided into four main groups i) branched *β*-(1,3) glucans with high molecular mass (pleuran, lentinan, grifolanand schizophyllan), ii) *β*-glucans with lower molecular mass (e.g. carboxymethyl glucan), iii) glucans with small molecule (e.g. zymosan) and iv) α-glucans [6,7].

β-glucans are natural components of cell walls of plants, yeast, algae, bacteria and mushrooms, consisting of a *β*-(1,3)-linked D-glucopyranosyl backbone to which either linked *β*-(1,6) or *β*-(1,4) side chains of varying distribution and length (Fig. 1-2). Cereal (e.g., barley and oats) and bacterial *β*-glucans are primarily linear with large regions of *β*-(1,4) linkages separating shorter stretches of *β*-(1,3) structures. Mushroom *β*-glucans have short *β*-(1,6)-linked branches coming from the *β*-(1,3) backbone, while those of yeast have *β*-(1,6) branches that are further elaborated with additional *β*-(1,3) regions (Table 1) [8,9].

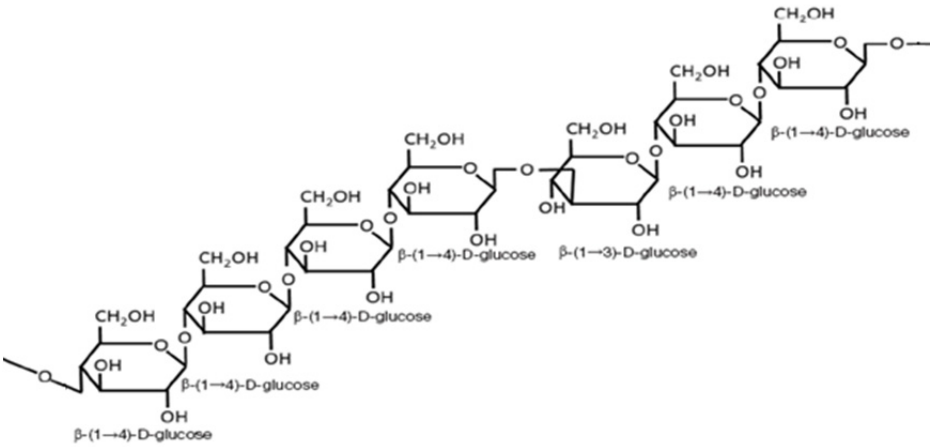


Fig. 1 Structure of *β*–glucans in cereals; *β*-(1,3) linear chain with *β*-(1,4) branch link bonds.

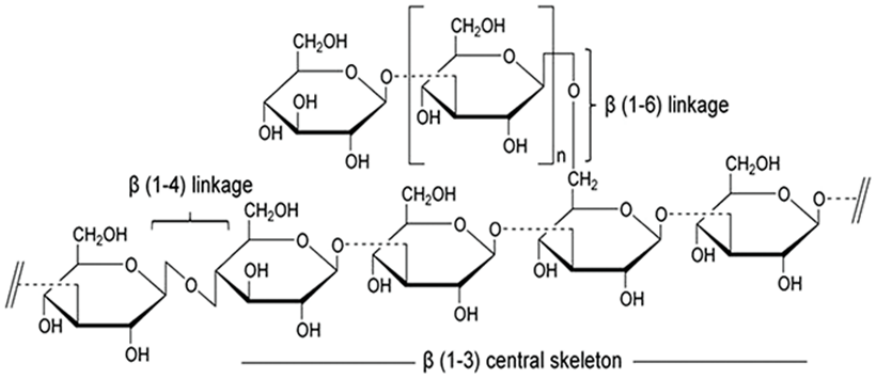


Fig. 2 Structure of *β*–glucans in yeasts; *β*-(1,3) linear chain with *β*-(1,6) branch link bonds.

The extraction conditions, molecular weight of *β*-glucans, degree of polymerization (DP) are responsible for their specific physical properties and characteristics, and determine their rheological properties and food applications [9-12].

Table 1 Beta-glucan types of different organisms with a schematic structure [11].

Organism	Structure	Description
Bacterial		Linear *β*-(1,3) glucan; i.e. curdlan
Yeast		Long chain *β*-(1,3) glucan with *β*-(1,6) branching; long spaces within branches; i.e. betafectini™, WGP *β*-glucan
Mushroom		Short chain *β*-(1,3) glucan with *β*-(1,6)branches; short spaces within branches; i.e. schizophyllan
Cereal		Linear *β*-(1,3)/ *β*-(1,4) glucan; i.e. oat, barley, rye

Table 2 Commonly used *β*-glucans [5,7,9,12]

Name	Source	Source type	Structure
Barley, oat, wheat, rye, rice		Cereal	*β*-(1,3)/*β*-(1,4) mixed linkage, unbranched
Curdlan	*Alcaligenes faecalis* *Agrobacterium radiobacter*	Gr (-) bacteria	*B*-(1,3) unbranched/ linear
Azorhizobium caulinodans	*A. caulinodans*	Gr (-) bacteria	*β*-(1,3)/*β*-(1,6) cyclic
Xanthan	*Xanthomonas campestris*	Gr (-) bacteria	*β*-(1,3)/ *β*-(1,4)/*β*-(1,2) side-chain-branched
Streptococcus pneumoniae	*Str. pneumoniae*	Gr (+) bacteria	*β*-(1,3)/*β*-(1,2) side-chain branched
CSBG	*Candida albicans*	Yeast	*β*-(1,3)/ *β*-(1,6) branched
Saccharomyces cerevisiae	*S. cerevisiae*	Yeast	*β*-(1,3) and small numbers of *β*-(1,6) branches
WPG-glucan (whole glucan particule)	*S. cerevisiae*	Yeast	*β*-(1,3)/*β*-(1,6) branched
Zymocel	*S. cerevisiae*	Yeast	Crude *β*-glucan extract
Zymosan	*S. cerevisiae*	Yeast	Crude extract with *β*-glucan and mannan non-uniform branches
GluP (fosforoglucan)	*S. cerevisiae*	Synthetic modified	*β*-(1,3) branched
PGG (betafektin)	*S. cerevisiae*	Genetically engineered	*β*-(1,3)/*β*-(1,6) highly branched
Glomerellan	*Glomerella cingulata*	Fungus	*β*-(1,3)/*β*-(1,6) branched
GRN (grifolan)	*Grifola frondosa*	Fungus/mushroom	*β*-(1,3)/*β*-(1,6) branched
LNT (lentinan)	*Lentinula edodes*	Fungus/mushroom	*β*-(1,3)/*β*-(1,6) branched
Pneumocytis carinii	*P. carinii*	Fungus/protozoan	*β*-(1,3)/*β*-(1,6) branched
P-SG (ganoderan)	*Ganoderma lucidum*	Fungus	*β*-(1,3)/*β*-(1,6) branched
SPG (sonifilan, schizophyllan)	*Schizophyllum commune*	Fungus	*β*-(1,3)/*β*-(1,6) branched
SR (skleoglucan)	*Sclerotium rolfssi* *S. glucanicum*	Fungus	*β*-(1,3)/*β*-(1,6) single branched
SSG	*S. sclerotium*	Fungus	*β*-(1,3)/*β*-(1,6) highly branched
LAM (laminarin, laminaran)	*Laminaria*	Algae i.e. brown seaweeds	*β*-(1,3) unbranched with some branching of *β*-(1,6)

β-glucans are commonly used, owing to their diverse physicochemical properties, to perform different functions in food technology, e.g. gel formation, foamability, stabilization, emulsification, water-binding and thickening. They are often used to improve the consistence of food products, e.g. drinks, dressings, fermented dairy products, dairy deserts or ice cream [13,14].

The molecular weights of *β*-glucans vary from tens to thousands of kilodaltons. The solubility of *β*-glucans in water is dependent mainly on their structure which is associated with their origin and increases with temperature. Protein-bound glucans are insoluble in water, only after partial hydrolysis, they produce gels. However, native *β*-glucan molecules lack this capability, so it can be said that *β*-glucans represent partly soluble and partly insoluble food ingredients [8].

β-glucan has been recognized to display many physiological functionalities and health benefits for humans; i.e. lowering blood glucose and insulin levels, reducing diabetic symptoms, improving lipid balance, reducing the risk of cardiovascular disease through its ability to lower serum cholesterol levels, and boosting the immune system and cancer prevention [15,16]. *β*-(1,3) (1-6)-*D*-glucans that have long, branched side chains are recognized as “good prebiotics”, which stimulate the growth of beneficial intestinal flora [17].

β-glucans are also used as a supplement to animal feed as an immune enhancer to limit infection occurrence, improve animal growth and development and decrease the use of antibiotics [5,18,19]. In addition, *β*-glucans are used not only in medicine but also employed in cosmetic industry, mainly for preparations to prevent irritation and delay skin aging [17].

2.2 *β*-glucan from cereals

β-glucans, appear mostly in the cell walls of the aleurone, sub-aleurone and endosperm tissues of cereals, consisting of unbranched *β*-glucans with glucopyranose molecules linked by *β*-(1,3)- and *β*-(1,4) linkages, and has been widely used in food products due to the health-enhancing benefits. The content of *β*-glucans in cereal grains depends on the source (cereal species, cultivation conditions), processing treatments (milling, temperature-pH-shear effects, etc.) and

interactions with other constituents (polymers or small molecular weight solutes) in the primary source or in a composite food matrix, of which designate the structural features and dispersibility-solubility of *β*-glucans, and thereby, modulate their physiological action in the gastro-intestinal tract [6,10]. The amount of *β*-glucans vary within the cereals, i.e. it is higher in barley (5-11%) than oats (3-7%) and wheat (0.2-1%) [2,6].

In barley *β*-glucan is mainly located in the endosperm and alerurone layer, which are consisted of 75% *β*-glucan + 20% of arabic gum and 26% *β*-glucan + 67% arabic gum, respectively. Wheat bran, consists of the outer coat (pericarp, testa and aleuron layers) of the wheat grain, is the main by-product of wheat milling, comprises approximately 6% *β*-(1,3)/*β*-(1,4)-glucan, used as the major resource for *β*-glucan extraction. However, the wheat aleurone layer contains 25% *β*-glucan. Thus, most of the recent research focuses on wheat kernel and wheat flour [20-21]. Biorklund et al. [22] stated that oat derived *β*-glucans, with high degree of water solubility, decreased significantly glycaemia and insulinemia than barley derived *β*-glucans that comprise lesser amount of water soluble components.

2.3 *β*-glucan from microorganisms

In general, in yeasts and other fungal cell walls *β*-glucans consist of glucopyranose molecules linked by *β*-(1,3) glycosidic linkages and small number of branches bound by 1,6-*β* bonds, whereas bacteria-derived *β*-glucans are not branched and include glucopyranose molecules linked by 1,3-*β* bonds [6].

A variety of bacteria, including important pathogens of humans, livestock and plants, produce extracellular and capsular polysaccharides (EPS), i.e. xanthan, dextran, pollulan and gellan, which are used on large scale in food industry for gelling, stabilizing and foaming properties [23]. These *β*-glucans are excreted from various microorganisms, such as *Pneumocystis carinii, Cryptococcus neoformans, Histoplasma capsulatum, Bacillus curdlanolyticus*, *B. kobensis*, *Sarcina ventriculi, Micromonospora, Agrobacterium* and *Rhizobacterium* [5,24-26].

Curdlan, isolated from *Agrobacterium* spp. and *Alcaligenes faecalis* var. *myxogenes*, exculisively containing *β*-(1,3)-glycosidic linkages, is a linear compound having a triple-helix conformation and a colourless and odourless compound which has been described as immunamodulator, anti-tumorogenic and anti-viral agent. It is insoluble in cold water, has unique rheological and thermal gelling properties and mainly used as a carrier for immobilized enzymes [27].

Fungi are an attractive source of physiologically functional foods and drug precursors, displaying a wide range of pharmacological activities such as anti-inflammatory, anti-tumor and immuno-modulating effects. The polysaccharide *β-D*-glucans are components that are found in some macrofungi (such as mushrooms), and unicellular fungi such as yeast (baker's yeast and *Candida albicans*) [28]. About half the mass of the fungal cell wall consists of *β*-glucans, but many are also excreted into the growth medium, making their recovery, purification and chemical characterization much simpler besides application in food and pharmaceutic industry [29]. Glucans differ in the structure of their side chains, water solubility, molecule size and molecular mass which are specific to the individual fungal species. The yeast and fungal glucans, either in water-soluble or insoluble forms, share the common structure: primary backbone chains of *β*-(1,3)-linked-glucopyranosyl units, along with randomly dispersed side chains of *β*-D-glucopyranosyl units attached by (1,6) linkages. These *β*-(1,3) (1,6)-glucans are usually highly branched; often present as an inner wall layer and are sometimes covalently associated with other cell wall polymers [30].

In yeasts cells the amount of *β*-glucans is higher than most cereal grains and vary between 29 and 64% depending on the cultivation conditions. Among yeasts, *Sacchromyces cerevisiae* is the major source of *β*-glucans comprising 55–60%. Other sources are *Zygosaccharomyces bailii*, *Kloeckera apiculata, Kluyveromyces marxianus, Debaryomyces hansenii,* and *Schizosaccharomyces pombe*. These *β*-glucans are built of glucose backbone with *β*-1,3 linkages, from which short side-chains branch off linked by *β*-(1,6) bonds [31].

Eventhough yeast-derived *β*-glucans are stated as being insoluble in water because of chitin, Akramiene et al. [26] pointed that *β*-glucans in inner layer of yeast cell wall are insoluble whereas the ones in outer layer are soluble. The insoluble *β*-glucans can induce immunity against bacterial, fungal, viral and parasite infections and against tumour cells [32], whereas the soluble form shows antioxidative properties [33]. Zymosan, derived from *Sacchromyces cerevisiae*, is an insoluble polymer of glucose, which demonstrates high antibacterial properties, induces inflammatory response and enhances immune system through activation of macrophages and induction of cytokine secretion [34].

Natural products containing fungal *β*-glucans have been consumed for probably thousands of years not only because of their nutritive but also because of improving general health and healing properties, especially in China and Japan [35,36]. The cell walls of macrofungi contain two polymers: chitin and *β*-glucan, in which individual chains are linked with hydrogen bridges and covalent bonds are formed between both polymers. Species of macrofungi used in ethnic medicine of the Far East are, *Ganoderma lucidum*, *Lentinula edodes*, *Grifola frondosa*, *Hericium erinaceus*, *Trametes versicolor*, *Schizophyllum commune*, *Phellinus linteus*, *Inonotus obliquus, Pleuortus ostreatus, Hirneola auricula*, *Stropharia aeruginosa*, *Agrocybe aegerita, Lyophyllum decastes, Calocybe indica, Armillaria melle* and *Collybia dryophila* [37,38].

The content of active substances depends on species, cultivation conditions, morphological stage (vegetative or reproductive) or part, maturation phase of fruiting bodies, storage conditions and processing methods [39,40]. In some members of the genus *Boletus β*-glucans comprise 2–13% of digestible dry matter [39], whereas in *Ganoderma lucidum* and Lingzhi mushroom *β*-glucan content is between 10 and 50% [41].

3. Physiological and health benefits of *β*-glucans

Many studies have indicated the beneficial health promoting effects of *β*-glucans, valuable functional ingredients, such as to lower the glucose and cholesterol levels in the serum, constipation relief, reduction of the risk of colorectal cancer, stimulation of the immune function, production of short chain fatty acids (SCFA) to promote the growth of beneficial gut microflora, prevention of coronary heart disease and diabetes, and increase the immunity to bacterial and parasitic infections [16,21,41]. The effect of decreasing blood glucose levels has been related to its property to form an unstirred water layer, which by resisting the convective effects of intestinal contractions, decreases sugar absorption by the small intestine [8,43]. Antitumour activity of *β*-glucans refers mainly to *β*-(1,3) (*1*,*6*)-form which has the ability to neutralise the free radicals, the vital reason of cancer occurrence [29].

It was reported that oat *β*-glucan could affect the upper part of the gastrointestinal tract of humans. After ingestion *β*-glucan starts to bind water, swell, form a gel-like network, dissolve in relationship to its size and previous hydrothermal treatments, and change the viscosity of gastrointestinal fluid. The increased volume causes a distension of the stomach, affects the satiety and reduces the rate of gastric emptying [12,44]. In the small intestine of humans, partly depolymerized during gastric ingestion, *β*-glucan remains intact, since no mammalian enzymes are capable of hydrolyzing it, and thereby act as a substrate for colon fermentations. As a prebiotic *β*-glucan affects the host by selectively stimulating the growth and/or activity of one strain or a limited number of bacterial strains in the colon, and thus, improves host health [45]. In the large intestine *β*-glucan behaves as a substrate, favoring production of SCFAs due to the oligosaccahrides favoring growth of some bacterial strains, enhancing production of microbial mass with good water retention properties, partly by its bulking effect [46].

Zhang et al. [47] reported that similar to other cereal-derived *β*-glucans, oat *β*-glucan, increased the insulin sensitivity index. As a soluble fiber of viscous characteristics it modifies the properties of chyme in the upper part of the gastrointestinal tract affecting gastric emptying, gut motility, and nutrient absorption, which are reflected in lower postprandial glycemic and insulin responses, which make *β*-glucan uptake beneficial for healthy subjects and patients with type-2 diabetes [48,49]. Since *β*-glucans decrease the blood cholesterol, improve the rheological property of blood, decrease the consumption of hepatic glucogen to increase the metabolism rate of glucose, and reduce the blood glucose level, due to the viscosity caused by the fiber and delay in intestinal absorbtion of carbohydrates it could have high significance in control and prevention of type-2 diabetes [50].

Due to the *β*-(1,3)-glycosidic bond, barley *β*-glucan has been reported to be capable of preventing cancer, reducing total serum cholesterol and low-density lipoprotein cholesterol (LDL) while increasing the high-density lipoprotein (HDL) cholesterol. Therefore, blends of food supplements containing barley *β*-glucan for specific health needs, mainly for prevention, treatment and control of diabetes, have been commercialized, such as pills, tablets and powders [9,51,52].

Some fungi-derived *β*-glucans, e.g. lentinan, schizophyllan, crestin, PSP and Grifron-D, are used in pharmaceutical preparations as antibacterial, antiviral and antiallergic agents [53,54] besides mentioned as having strong antioxidative properties [15]. As much as fungi-derived *β*-glucans may have stimulatory effects on the immune system, leading to resistance against viral, bacterial, parasitic, and fungal pathogens, the cereal-derived *β*-glucans have also been ascribed to have immune-stimulating properties. It was reported that natural *β*-glucans have antibiotic potential by increasing bacterial clearance, increasing bactericidal activity, increasing modulation of cytokine production, and increasing the number of monocytes and neutrophils [42,55].

In humans, fungal *β*-glucans, as for cereal *β*-glucans, have been shown to reduce both the overall level of cholesterol and the level of LDL cholesterol in blood. Lower levels of cholesterol is probably correlated with increased amounts of leptin, a protein-like substance, which is produced by fat cells of the subcutaneous connective tissues and is commonly found in blood. Since leptin control the feelings of hunger and satiety, it could be said that leptin mediates the feedback of fat content within the body and may offer a promising way to control obesity [56].

4. Industrial applications of *β*-glucans

Food industry may take the advantage of *β*-glucan, functional ingredient, having a growing market. Consumer demand for healthy and nutraceutical food is the major motive for this growth. However, to be used in products that offer a wide range of added health benefits its purity should be at high level In this context, *β*-glucans, extracted from different sources, have been marketed in various forms such as *β*-glucan concentrate extracted from oats (Oatrim™), *β*-glucan from barley (NutrimXe™) and *β*-glucan extracted from rice (Ricetrim™) [28,57].

Recent research is focused on exploration of the ways to incorporate *β*-glucans into various food systems, the emphasis mainly being on the rheological properties of *β*-glucan in water solution and viscoelasticity change under different food processing conditions. *β*-glucan has various applications in the food process industry as thickening, stabilizing, emulsification, and gelation agents to increase viscosity, substitute fat, and improve rheological properties in soups, sauces, beverages, and in other food products [13,58]. The functional properties of *β*-glucan, such as viscosity, foaming stability and emulsifying property, are affected mainly by the structure, molecular weight, conformation, temperature, and pH. The rheological properties of *β*-glucans are also correlated to their physiological properties. *β*-

glucan has high water holding capacity and gelling property. When dissolved in water, *β*-glucan form a viscous solution [2] of which the viscosity increases as the concentration of the solution or the molar mass of *β*-glucan increases [59].

Autio et al. [60] found that *β*-glucan exhibited good homogeneous property and viscous fluid characteristics when the concentration was below 1%; while the concentration increased to 2%, *β*-glucan performed heterogeneous property and viscoelasticity. All the changes happened in a very narrow range of concentration between 1–2%, which could be attributed to the strong interaction between the chains and aggregation at high concentration.

Effects of *β*-glucan on gelation and rheological characteristics of dairy-based products such as in yogurt [2,61-63], dairy gels [64] and cheese [65,66], low-fat ice creams [67], and low-fat cheese curds [68] have been investigated. Even dairy products are ascribed as not being a good source of fiber, they could serve as an alternative vehicle for the development of fiber-enriched foods. Sharafbafi et al. [69] incorporated high-molecular weight oat *β*-glucan into milk to obtain calorie-reduced and cholesterol-lowering dairy products, and the phase behavior, rheological properties, and microstructure of this dairy product were analyzed. The results showed that the flow behavior of the mixtures with concentrations higher than the binodal curve was not only governed by the presence of *β*-glucan chains, but also by the formation of these structures.

Its properties enable it to be incorporated alternatively in beverages as replacement for thickeners such as gum arabic, alginates, pectin, and carboxymethyl-cellulose [70]. Barley *β*-glucan is particularly well suited for such applications in beverages, as being capable of imparting a smooth mouth feel. The final product is described as an excellent source of soluble dietary fiber [7].

5. Conclusion

Currently numerous researches are being conducted on the impact of *β*-glucans on human health and its use in various foods and dairy products. The *β*-glucan from various resources have different molecular structures and thus display different physiochemical characteristics and rheological properties, which designate and limit their use in various food systems. However, due to the mentioned health benefits as a dietary fiber and textural impact on food systems, future research should aim utilization of *β*-glucans for the development of novel functional products.

References

[1] Turner ND, Lupton JR. Dietary Fiber Advances in Nutrition: An International Review of Journals. 2011; 2:151-52.

[2] Ozcan T, Kurtuldu O. Influence of dietary fiber Addition on the properties of probiotic yogurt. International Journal of Chemical Engineering. 2014; 5:397–401.

[3] Velasquez M, Davies C, Marret R, Slavin JL, Feirtag JM. Effect of oligosaccharides and fibre substitutes on short chain fatty acid production by human microflora. Anaerobe. 2000; 6:87–92.

[4] Crittenden RS, Karppinnen S, Ojanen M, Jenkanen J, Mattila S, Poutanen K. In vitro fermentation of cereal dietary fibre carbohydrates by probiotic and intestinal bacteria. Journal of the Science Food and Agriculture. 2002; 82:781–89.

[5] Havrlentova M, Petrulakova Z, Burgarova A, Gago F, Hlinkova A, Sturdik E. Cereal β-glucans and their significance for the preparation of functional foods: A review. Czech Journal of Food Sciences. 2011; 29: 1–14.

[6] Laroche C, Michaud P. New developments and prospective for *β*-(1,3) glucans. Recent Patents on Biotechnology. 2007; 1:59–73.

[7] Ahmad A, Anjum FM, Zahoor T, Nawaz H, Dilshad SM. Beta glucan: A valuable functional ingredient in foods. *Critical Reviews and Food Science.* 2012; 52:201–212.

[8] Rop O, Mlcek J, Jurikova T. Beta-glucans in higher fungi and their health effects. Nutrition Reviews. 2009; 67:624–31.

[9] Zhu FM, Du B, Bian ZX, Xu BJ. Beta-glucans from edible and medicinal mushrooms: characteristics, physicochemical and biological activities. Journal of Food Composition and Analysis. 2015; 41:165–173.

[10] Skendi A, Biliaderis CG, Lazaridou A, Lzydorczyk MS. Structure and rheological properties of water soluble β-glucans from oat cultivars of Avena sativa and Avena bysantina. Journal of Cereal Science. 2003; 38:15–31.

[11] Volman JJ, Ramakers JD. Plat J. Dietary modulation of immune function by *β*-glucans. Physiology & Behavior. 2008; 94:276–84.

[12] Daou C, Zhang H. Oat beta-glucan: its role in health promotion and prevention of diseases. Comprehensive Reviews in Food Science and Food Safety. 2012; 11:355–65.

[13] Dongowski G, Drzikova B, Senge B, Blochwitz R, Gebhardt E, Habel, A. Rheological behaviour of *β*-glucan preparations from oat products. Food Chemistry. 2005; 93:279–91.

[14] Worrasinchai S, Suphantharika M, Pinjai S, Jamnong P. β-glucan prepared from spentbrewer's yeast as a fat replacer in mayonnaise. Food Hydrocolloids. 2006; 20:68–78.

[15] Tsiapali E, Whale YS, Kalbfleisch J, Ensley HE, Browder IW, Williams DL. Glucans exhibit weak antioxidant activity, but stimulate macrophage free radical activity. Free Radical Biology & Medicine. 2001; 30:393–402.

[16] Queenan KM, Stewart ML, Smith KN, Thomas W, Fulcher RG, Slavin JL. Concentrated oat *β*-glucan, a fermentable fiber, lowers serum cholesterol in hypercholesterolemic adults in a randomized controlled trial. Nutrition Journal. 2007; 6:1–8.

[17] Szymańska-Czerwińska M, Bednarek D. Beta-glukany alternatywą antybiotykowych stymulatorów wzrostu. Życie Weterynaryjne. 2007; 82:842–43.

[18] Petravic-Tominac V, Zechner-Krpan V, Grba S, Srecec S, Panjkota-Krbavcic I, Vidovic L. Biological effects of yeast β-glucans. Agriculturae Conspectus Scientificus. 2010; 75:149–58.

[19] Sobieralski K, Siwulski M, Lisiecka J, Jedryczka M, Sas-Golak I, Frużyńska-Jóźwiak D. Fungi-derived *β*-glucans as a component of functional food. Acta Scientiarum Polonorum Hortorum Cultus. 2012; 1:111–28.
[20] Wang N, Zhao M. Study progress on *β*-glucan in barley. Science Technology Qinghai Agricultural For. 2007; 3:34–6 (in Chinese).
[21] Yu L, Tsao R, Shahidi F. Cereals and pulses: Nutraceutical properties and health benefits. In: Jing P, Hu XZ editors. International Nutraceutical Properties and Health Benefits of Oats. John Wiley & Sons, Inc, Ames, IA; 2012. p. 221–40.
[22] Biorklund M, van Rees A, Mensink R, Onning G. Changes in serum lipids and postprandial glucose and insulin concentrations after consumption of beverages with β-glucans from oats or barley, a randomized dose-controlled trial. European Journal of Clinical Nutrition. 2005; 59:1272–81.
[23] Funane K, Ishii T, Matsushita M, Hori K, Mizuno K, Takahara H, Kitamura Y, Kobayashi M. Water-soluble and water-insoluble glucans produced by *Escherichia coli* recombinant dextransucrases from *Leuconostoc mesenteroides* NRRL B-512F. Carbohydrate Research. 2001; 334:19–25.
[24] Stasinopoulos SJ, Fisher PR, Stone BA, Stanisich VA. Detection of two loci involved in (1→3)-β-glucan (curdlan) biosynthesis by *Agrobacterium* sp. ATCC31749, and comparative sequence of the putative curdlan synthase gene. Glycobiology. 1999; 9:31–41.
[25] Obst M, Sallam A, Luftmann H, Steinbuchel A. Isolation and characterization of grampositivecyanophycin-degrading bacteria-kinetic studies on cyanophycin depolymerase activityin aerobic bacteri. Biomacromolecules. 2004; 5:153–61.
[26] Akramiene D, Kondrotas A, Didziapetriene J, Kevelaitis E. Effects of *β*-glucans on the immune system. Medicina (Kaunas). 2007; 43:597–606.
[27] Williams PD, Sadar LN, Lo YM. Texture and stability of hydrogel complex containing curdlan gum over multiple freeze-thaw cycles. Food Processing and Preservation. 2009; 33:126–39.
[28] Du B, Lin C, Bian Z, Xu B. An insight into anti-inflammatory effects of fungal beta-glucans. Trends in Food Science & Technology. 2015; 41:49–59.
[29] Chen J, Seviour R. Medicinal importance of fungal *β*-(1-3), (1-6)-glucans. Mycological Research. 2007; 111:635–652.
[30] Zlatkovic D, Jakovljevic D, Zekovic D, Miroslav MV. A glucan from active dry baker's yeast (Saccharomyces cerevisiae): a chemical and enzymatic investigation of the structure. Journal of the Serbian Chemical Society. 2003; 68:805–809.
[31] Saluk-Juszczak J, Królewska K. β-glucan drożdży *Saccharomyces cerevisiae* – naturalnystymulator układu immunologicznego. Probemy Nauk Biologicznych. 2010; 59:151–160.
[32] Yoon TJ, Kim TJ, Lee H, Shin KS, Yun YP, Moon WK, Kim DW, Lee KH. Antitumormetastatic activity of β-glucan purified from mutated *Saccharomyces cerevisiae*. International Immunopharmacology. 2008; 8:36–42.
[33] Sandula J, Kogan G, Kacurakova M, Machova E. Microbial (1→3)-β-D-glucans, theirpreparation, physico-chemical characterization and immunomodulatory activity. Carbohydrate Polymers. 1999; 38: 247–253.
[34] Brown GD, Herre J, Wiliams DL, Willment JA, Marshall AS, Gordon S. Dectin-1 mediates the biological effects of beta-glucans. The Journal of Experimental Medicine. 2003; 197:1119–1124.
[35] Zaidman BZ, Yassin M, Mahajna J, Wasser SP. Medicinal mushroom modulators ofmolecular targets as cancer therapeutics. Applied Microbiology and Biotechnology. 2005; 67:453–68.
[36] Guillamon E, Garcia-Lafuente A, Lozano M, D'arrigo M, Rostagno MA, Villares A, Martinez JA. Edible mushrooms, role in the prevention of cardiovascular diseases. Fititerapia. 2010; 81:715–23.
[37] Reverberi M, Di Mario F, Tomati U. Beta-glucan synthase induction in mushrooms grown on olive mill wastewaters. Applied Microbiology and Biotechnology. 2004; 66:217–25.
[38] Sanchez-Pacheco M, Boutin Y, Angers P, Gosselin A, Tweddell RJ. A bioactive (1→3), (1→4)- β-D-glucan from *Collybia dryophila* and other mushrooms. Mycologia. 2006; 98:180–185.
[39] Manzi P, Marconi S, Aguzzi A, Pizzoferrato L. Commercial mushrooms, nutritional quality and effect of cooking. Food Chemistry. 2004; 84:201–06.
[40] Kimmons TE, Phillips M, Brauer D. Effects of Management Factors on the concentration of a high molecular weight polysaccharide fraction from log-grown shiitake mushrooms (*Lentinula edodes*). Journal of Agricultural and Food Chemistry. 2010; 58: 4331–35.
[41] Wasser SP. Current finding, future trends, and unsolved problems in studies of medicinal mushrooms. Applied Microbiology and Biotechnology. 2011; 89:1323–32.
[42] Yun CH, Estrada A, Van Kessel A, Park BC. Laarveld B. Beta-glucan, extracted fromoat, enhances disease resistance against bacterial and parasitic infections. FEMS Immunology and Medical Microbiology. 2003; 35:67–75.
[43] Perera PK, Li Y. Mushrooms as a Functional food mediator in preventing and ameliorating diabetes. Functional Foods in Health and Disease. 2011; 4:161–71.
[44] Mälkki Y, Virtanen E. Gastrointestinal effects of oat bran and oat gum: A review. Lebensm. -Wiss. U.-Technology. 2001; 34:337–47.
[45] Gibson GR, Roberfroid B. Dietary modulation of the human colonic microflora: introducing the concept of prebiotics. The Journal of Nutrition. 1995; 125:1401–12.
[46] Bach Knudsen KE, Hansen I, Borg JB, Ostergard K. Physiological implications of wheat and oat dietary fibre. In: Furda I, Brine CJ, editors. New developments in dietary fiber. New York: Plenum Press; 1990. p. 135–50.
[47] Zhang PP, Hu XZ, Xu C, Zhen HM, Fan MT. Oat β-glucan increased ATPases activity and Energy insmall intestine of rats. Journal of Agricultural Food Chemistry. 2012; 60:9822–27.
[48] Charles SB. Dietary fiber, glycemic response, and diabetes. Molecular Nutrition & Food Research. 2005; 49:560–70.
[49] Behall KM, Scholfield DJ, Hallfrisch JG. Barley *β*-glucan reduces plasma glucose and insulin responses compared with resistant starch in men. Nutrition Research. 2006; 26:644–50.
[50] Wood, PJ, Braaten, JT, Scott, FW, Riedel, KD, Wolynetz, MS, Collins, MW. Effect of dose and modification of viscous properties of oat gum on plasma glucose and insulin following an oral glucose load. British Journal of Nutrition. 1994; 72:731–43.

[51] Rahar S, Swami G, Singh GS. Preparation, characterization, and biological properties of β-glucans. Journal of Advanced Pharmaceutical Technology & Research. 2011; 2: 94-103.
[52] Brennan CS, Cleary LJ. The potential use of (1/3, 1/4)-*β*-D-glucans as functional food ingredients. Journal of Cereal Science. 2005; 42:1–13.
[53] Smith JE, Rowan NJ, Sullivan R. Medicinal mushrooms, a rapidly developing area of biotechnology for cancer therapy and other bioactivities. Biotechnology Letters. 2002; 24:1839–45.
[54] Kumar CG, Joo HS, Choi JW, Koo YM, Chang CS. Purification and characterisationof an extracellular polysaccharide from haloalkalophilic *Bacillus* sp. I-450. Enzyme and Microbial Technology. 2004; 34: 673–81.
[55] Kaiser AB, Kernodle D. Synergism between poly-(1–6)-*β*-D-glucopyranose glucana and cefazolin in prophylaxis of staphylococcal wound infection in guinea pig model. Antimicrob Agents Chemother. 2002; 42:2449–51.
[56] Prado WL, Balagopal PB, Lofrano-Prado MC, Oyama LM, Tenório TR, Botero JP, Hill JO. Effect of aerobic exercise on hunger feelings and satiety regulating hormones in obese teenage girls. Pediatric Exercise Science. 2014; 26:463-69.
[57] Inglett GE, Carriere CJ, Maneepun S, Tungtrakul P. A soluble fiber gel produced from rice bran and barley flour as a fat replacer in Asian foods. International Journal of Food Science and Technology. 2004; 39:1–10.
[58] Burkus Z, Temelli,F. Stabilization of emulsions and foams using barley beta-glucan. Food Research International. 2000; 33:27–33.
[59] Wang HB, Xu QY, Liu DC,Wang HY, Xi, BJ. Rheological properties of β-glucans from oats. English Abs of Transactions from the Chinese Society of Agricultural Engineering. 2008; 24:31–5.
[60] Autio K, Myllymäki O, Mälkki Y. Flow properties of solution of oat *β*-glucan. Food Science. 1987; 52: 1364–66.
[61] Vasiljevic T, Kealy T, Mishra VK. Effects of β-glucan addition to a probiotic containing yogurt. Journal of Food Science. (2007); 72:405–11.
[62] Sahan N, Yasar K, Hayaloglu AA. Physical, chemical and flavour quality of non-fat yogurt as affected by a β-glucan hydrocolloidal composite during storage. Food Hydrocolloids. 2008; 22:1291–97.
[63] Rosburg V, Boylston T, White P. Viability of *Bifidobacteria* strains in yogurt with added oat beta-glucan and corn starch during cold storage. Journal of Food. 2010; 75:439-44.
[64] Sharafbafi N. Structuring Properties of Beta-glucan in Dairy Gels: Control of Phase Separation, A thesis presented to The University of Guelph, 2012; p.119.
[65] Konuklar G, Inglett GE, Warner K, Carriere CJ. Use of a *β*- glucan hydrocolloidal suspension in the manufacture of low-fat cheddar cheeses: textural properties by instrumental methods and sensory panels. Food Hydrocolloids. 2004; 18:535–545.
[66] Volikakis P, Biliaderis CG, Vamvakas C, Zerfridis GK. Effects of a commercial oat-*β*-glucan concentrate on the chemical, physico-chemical and sensory attributes of low-fat white-brined cheese product. Food Research International. 2004; 37:83–94.
[67] Brennan CS, Tudorica CM, Kuri V. Soluble and insoluble dietary fibers (non-starch polysaccharides) and their effects on food structure and nutrition. International Journal of Food. 2002; 5:261–72.
[68] Tudorica CM, Jones E, Kuri V, Brennan CS. The effects of refined barley *β*-glucan on the physico-structural properties of low-fat dairy products: curd yield, microstructure, texture and rheology. Journal of Science Food and Agricultural. 2004; 84:1159–1169.
[69] Sharafbafi N, Tosh SM, Alexander M, Corredig M. Phase behaviour, rheological properties, and microstructure of oat *β*-glucan-milk mixtures. Food Hydrocolloids. 2014; 41:274–280.
[70] Ahmad A, Anjum FM, Zahoor T, Nawaz H, Ahmed Z. Extraction and characterization of *β*-D-glucan from oat for industrial utilization. International Journal of *Biological* Macromolecules. 2010; 46:304–309.

The role of saliva in food sensory perception: relevant knowledge to design healthy foods

Elsa Lamy[1], **Lénia Rodrigues**[1], **Teresa Louro**[1] and **Fernando Capela e Silva**[1,2]

[1] Institute of Mediterranean Agricultural and Environmental Sciences (ICAAM), University of Évora, Largo dos Colegiais 2, 7000 Évora, Portugal

[2] Department of Biology, School of Science and Technology, University of Évora, Largo dos Colegiais 2, 7000 Évora, Portugal

Food choices and consumption are determined by a range of factors that contribute to aversion or pleasure and guide to final intake. Among these, the sensorial characteristics of food have a major and decisive role in choice behaviour. Although some of the mechanisms involved in oral food perception, namely in taste and astringency perception, are considerable known, many questions remains, particularly in what concerns variations among individuals in their sensitivity for food sensorial aspects. The understanding of the mechanisms leading to different responses for the same sensorial stimulus is particularly important to understand food choices.

Bitter has been the basic taste most studied for variations among individuals in perception and in how this influences food behaviour and nutritional status. The observation, at several years ago, that some individuals are very sensitive to the bitterness of the compounds phenyl thiocarbamide (PTC) or 6-n-propylthyouracil (PROP), whereas others are almost insensitive, triggered the emergence of diverse studies about the motif for that, resulting in the identification of gene polymorphisms for the bitter taste receptor TAS2R38. Subsequently to that, polymorphisms for other receptors and taste qualities have been identified. Even so, these genetic variations are not able to explain the total diversity in taste/oral sensations responses. In recent years, it has begun to become apparent that saliva has a relevant role in taste recognition mechanisms. Apart from astringency, which is well known to depend on salivary proteins to develop and being perceived, basic tastes started to be related with saliva composition. Some salivary proteins, among which carbonic anhydrase VI, cystatins, amylase and others, have been observed to relate with taste perception. However, saliva secretion changes with taste stimulation and according dietary habits. Moreover, body weight condition, metabolic status or diverse pathologies are responsible for changes in saliva composition. Being this fluid important in modulating oral food perception, to know individuals' saliva composition becomes of interest for modulating or directing choices. Based on the literature and recent scientific results, the role of saliva in food sensory perception will be discussed according to these two angles. The question of the high between-subject variability in view of saliva properties and its consequence on perception will be emphasized.

Keywords: Saliva, oral sensory perception, healthy foods

1. Introduction - Determinants of food choices

All of us need to eat, but each of us have different preferences and make different choices whenever possible. These different choices result in different dietary styles, which can have influence in health. The understanding of the factors influencing ingestive behaviour is fundamental for avoidance and resolution of numerous nutritional problems, as well as for food industry development and promotion of products. Consumer choices are influenced by diverse factors, among which biological and sensory attributes play a key role. Besides these, food choices and intake also depend on psychological and social factors, including beliefs, habits, values and past experiences [1]. Aspects such as age, gender, individual's personality, different levels of knowledge and experience with regard to food related issues may induce different types of behaviours relative to food [2]. Moreover, an individual's thought about food will influence sensorial perception of that food. For example, familiar brands can modulate individual's taste perception [3]. As well, an apparently unrelated cue, such as the sound, may change the way food is perceived in the mouth. This is observed by famous chefs, such as Heston Blumethal, in its daily contact with his restaurant consumers, who report that the salty taste of a seafood dish is higher when eaten at the same time that the sound of sea is listen. The study of the effect of sound in consumer food perception has gained interest in the last years [4].

The accessibility of resources is another important aspect when choosing food. The higher consumption of fish by the populations that lived near sea or vegetables from rural individuals are good examples of intake based on products availability. This is not so evident nowadays, in developed countries, where supermarkets contain products from a diversity of places. Even so, it is still possible to observe some different food habits from rural populations, comparatively to urban ones. Low financial resources, transportation constraint and architectural barriers, may also prevent people from having access to preferred food products and determine the purchase of more or less suitable alternatives, which in turn may influence habitual preferences [5].

Concerning biological factors, sensory-affective responses to food are a major influence on food preferences and choices [6]. Palatability links sensorial and physiological cues with the emotional perception of foods and is a major factor in food acceptance and choices. Food sensorial characteristics such as taste, texture, smell and appearance

influences palatability and, as such, the perception of food sensorial properties is one of the main determinants of food consumption.

Among the sensorial characteristics, taste and smell are chemical senses of well-known influence in food perception and choices. Inter-individual differences in taste perception have been studied and may explain some of the differences in food acceptance and choices.

This chapter presents a review about how oral perception relates with food acceptance and choices. Particular attention will be given to saliva due to the recent evidences of its influence in food sensorial perception, namely in taste and astringency perception. The way this may influence sustainable and healthy choices will be discussed.

2. Oral food perception

2.1 Taste system: anatomy and physiology

At the moment, five basic tastes are accepted: sweet, sour, bitter, salty and umami. In the recent years more sensations have been proposed as basic tastes, namely the taste of fat [7] and the taste of carbohydrates [8]. Taste perception is important to ensure the acquisition of nutrients and minerals and to avoid the intake of potentially nocive substances. Aversion caused by sourness and bitter taste, for example, prevents mammals to ingest injurious food substances, whereas sweetness of sugars guides mammals to ingest energy [9].

Non-volatile food constituents, dissolved in saliva, are detected by receptors present in taste cells, which are embedded in structures called taste buds, located in tongue, soft palate, epiglottis, larynx, and pharynx. Taste buds consist in groups of 50-100 cells, which are divided in four types of cells (types I to IV), which have been described based on their ultrastructural and cytological characteristics: type I cells appear to be supporting cells; type II are the taste receptor cells; type III are cells with characteristics that are “intermediate” between type I and type II cells and form synapses with afferent nerve fibres; type IV cells are basal cells, which are thought to have the capacity to differentiate and replace the cells in taste buds (reviewed in [10]).

Food molecules, responsible for taste, contact with taste cells through the taste pore located in the apical region of taste buds. Most of the information about taste starts with the contact of these molecules with the microvilli of these cells, leading to intra-cellular signal transduction. Moreover, the transduction machinery also involves ion channels on both the apical and basolateral membrane. When chemical stimuli interacts with taste cells, voltage-gated Na+, K+ and Ca2+ channels, located on the basolateral membrane of these cells, produce depolarizing potentials. This raise Ca2+ levels, leading to synaptic vesicle fusion and synaptic transmission [11]. The information goes from taste buds to the brain via afferent fibres from branches of three cranial nerves: VII, IX and X cranial nerves. Taste information is transmitted into the brain stem (nucleus tractus solitarius - NTS), from this to the ventroposteromedial nucleus of thalamus and from this to insular/opercular cortex (primary gustatory cortex) and orbitofrontal cortex (secondary gustatory cortex).

2.2 Factors influencing taste sensitivity

Although sweetness is universally accepted and preferred and bitterness avoided, the concentration at which these senses result in such responses varies according to the molecule that induce each of them, to the matrix in which that molecule is present (and the relative amount of different compounds that constitute that matrix) and to individual’s characteristics.

Taste sensitivity is by definition the ability with which each individual perceives the different tastes. The inter-individual variability in perception of basic tastes began to be noted for bitter taste. The notion that this taste is not sensed in the same way by all individuals arose in the thirties, of the last century, more or less "by chance." When weighing the compound phenylthiocarbamide (PTC) in the laboratory, some powders from this reagent were released, with some investigators "complaining" of bitterness, while others did not feel or felt with very little intensity of such sensation [12]. From then on, variations in bitterness perception started to be studied in detail, particularly for PTC and the other compound of the same family, 6-n-propyl-thiouracil (PROP). Several studies have been performed to elucidate the mechanisms underlying variation in bitterness. Three main factors have been proposed: i) specific genetic variation (polymorphisms present in taste receptors) [13]; ii) generic genetic variation (eg, taste bud density) [14]; iii) and environmental factors, such as eating habits [15] or even saliva composition [16–18].

The taste receptor that responds to PTC and PROP compounds is encoded by the TAS2R38 gene, for which polymorphisms have been identified and related to bitter taste perception [19]. These single nucleotide polymorphisms (SNPs) result in the substitution of three amino acids (Pro49Ala, Ala262Val and Val296Ile), giving rise to two common haplotypes: PAV, dominant variation, and AVI, recessive variation. Homozygous or heterozygous individuals for the haplotype PAV perceive the bitterness of PROP at low concentrations and are considered tasters (homozygous being super tasters and heterozygous medium-tasters), while individuals homozygous for the AVI haplotype do not perceive PROP bitterness or perceive it only when in high concentrations, being classified as non-tasters [13].

Some studies exist also for other basic tastes, where genetic factors have been reported as influencing sensitivity. Variation in the TAS1R2 gene, which codifies one component of the G-protein–coupled receptors TAS1R2-TAS1R3, has been linked to sweet taste sensitivity and food choices, although such relationship being dependent on Body Mass Index (BMI) [20]. For sour and salty taste, receptors are less well characterized. Even so, Dias and colleagues [21] reported single nucleotide polymorphisms in the putative salt taste receptors, ENaC and TRPV1, which were associated with differences in salt taste perception. The association between polymorphisms in taste receptors and taste perception and eating behavior has been recently reviewed [22].

Although genetic changes in the membrane receptor are an important factor, they do not fully explain the variations observed in taste sensitivity. According to Genick and colleagues [23], approximately 30% of the observed phenotypic variation in bitter taste response should be explained by other factors, such as changes in the characteristics of the oral environment involving receptors. In this context, saliva composition started to be taken into account, and nowadays there are evidences that this fluid is related to bitter (e.g. [16,24]) and sweet (Rodrigues et al., Salivary proteome and glucose levels are related with sweet taste sensitivity in young adults, *submitted*) taste sensitivities. More detail about this issue will be presented in the next point.

3. Saliva and oral food perception

3.1 Saliva composition and functions

Whole saliva, the fluid that continually baths the mucosa of the oral cavity, oropharynx and larynx is a complex mixture deriving from the secretion of salivary glands and gingival crevicular fluid, containing oral bacteria, food remainders and desquamated epithelial and blood cells [25]. Total saliva is composed by a variety of electrolytes, including sodium, potassium, calcium, magnesium, bicarbonate and phosphates, by proteins such as immunoglobulins, enzymes (e.g amylase and lysozyme), mucins (glycoproteins involved in the protection and prevention of oral epithelium), microbial enzymes and nitrogenous products such as urea and ammonia. The relative proportion of these different components varies with several factors, among which changes in salivary flow [25].

Saliva is an exocrine secretion of specialized cells grouped in salivary glands, distributed in the oral cavity. According to their size and contribution for the total amount of saliva, the salivary glands are classified as "major" and "minor". There are three pairs of major salivary glands (which exist as bilateral pairs): parotid, submandibular and sublingual glands. Of the major salivary glands, the parotid is the largest one and contributes with the greatest flow (as much as 60% of the total) when stimulated by taste or chewing [26]. However, this gland contributes with only a small amount to resting salivary flow. It secretes a serous secretion that contains no mucins but is rich in amylase and proline-rich proteins (PRPs) [27]. In addition to the major salivary glands, there are hundreds of minor salivary glands located in the submucosa throughout the oral cavity. Most of these glands produce a mucous secretion, in small volumes (<1 µl min^{-1} per gland) with mucin-rich content. Although only contributing approximately 10% of salivary flow, the minor glands are important in maintaining a mucin-rich layer adjacent to the mucosa [27].

There are two main types of salivary secretion: serous and mucous secretion. The first is a fluid secretion rich in water, with enzymes and diverse other proteins. It plays an important role in ingestion and chewing of food. Serous fluid is produced and secreted by the serous acinar cells of the parotid glands and some minor salivary glands, such as von Ebner's glands in tongue. Mucous secretion is rich in glycoproteins such as mucins, acting predominantly in lubrication, bolus formation and deglutition. This type of secretion is derived from mucosal cells. Glands such as the sublingual glands and several minor salivary glands are mostly (or even totally) constituted by this type of cells [28]. It is also possible to consider a third type of secretion, mixed between the two mentioned above, as is the case of the secretion coming from the submandibular glands, which are mixed glands, constituted both by serous and mucous cells.

Five main functions of saliva have been proposed: (1) lubrication and protection, (2) buffering and antibacterial activity, (3) maintenance of tooth integrity, (4) tissue repair and (5) taste and digestion [25]. The major lubricating components of saliva are mucins. Chewing, speech and swallowing are processes aided by the lubricating effect of these glycoproteins. Mucins also perform antibacterial activity through the selective modulation of microorganism adhesion to oral tissues, contributing to protection of oral cavity. The buffering capacity of saliva is mainly due to its composition in bicarbonates, phosphates and proteins. Moreover, saliva contains a spectrum of proteins with antibacterial properties, such as histatin and lysozyme, which can hydrolyse the cell wall of some bacteria [27]. The maintenance of tooth integrity is another of the functions performed by saliva. This fluid is saturated with calcium and phosphate ions, ensuring the ionic exchange directed to the tooth surface.

With regard to oral perception, saliva also plays an important role. This fluid is required to dissolve the substances which are then transported to the taste receiving/detecting sites (taste-receiving cells located in the taste buds) [29]. Moreover, salivary constituents may interact with food constituents, promoting modifications or changing its assessment to the structures responsible or sensorial detection, such will be subsequently detailed.

3.2 The role of saliva in oral perception and eating behavior

Several studies, in animals and humans, present evidences that saliva is involved in eating behaviour (e.g. [18,30–32]). The link between saliva composition and oral perception is increasingly reported. The involvement of salivary proteins in the perception of food has been most studied in the context of its effect on the development of astringency. Although the mechanisms involved in astringency development are not fully elucidates, the participation of salivary proteins is well accepted. One of the main accepted theories for explaining astringency is based in a two-phase model: a first phase, in which polyphenols (or other astringent molecules) bind to the proteins present in the "liquid part" of saliva; a second phase where the astringent molecules that did not bind in phase 1 can interact with the adsorbed glycoprotein layer, in the oral mucosa, reducing lubrication of the oral cavity and developing astringency. Different salivary proteins have been linked to the astringency caused by polyphenols, due to their affinity for these compounds. Among these, PRPs [33], histatins [34], cystatins [35] and α-amylase [36] have been observed to bind polyphenols. Salivary PRPs constitute the main family of salivary proteins that are associated with astringency. These proteins, due to the richness in the amino acid proline, have an open structure that allows them to bind tannins with high affinity, forming stable complexes [37]. Mucins also play a role in astringency, although there are some controversies in this regard. Studies developed by McColl and colleagues [38] present evidence that mucins have a reduced lubrication effect when mixed with tannins. Recently, it has been observed that astringent dietary components may influence the lubricating properties of the protective mucous barrier in the oral cavity, by affecting the arrangement of the salivary mucins MUC5 (gel forming) and MUC7 (non-gel-forming) [39].

Other salivary proteins have also been reported as potentially involved in oral sensorial perception, namely at taste level. Lipocalin 1 protein, originated from the Von Ebner glands [40], shows homology with transporters of hydrophobic molecules, and it has been proposed that they can assist in the concentration and transport of molecules to the taste receptor cells [41]. Carbonic anhydrase VI (CA-VI) is the salivary protein most reported as associated with bitter taste perception. Besides parotid, the Von Ebner glands, which are located near tongue circumvallate papillae, secretes this protein. The close proximity of von Ebner's glands with these taste papilla, which have high density of bitter taste receptors, lead to the suggestion that their secretion, including CA-VI, could affect directly bitter taste. CA-VI have been pointed as implicated in the growth and renewal of taste buds, since the levels of this protein have been observed to be reduced in individuals with taste buds anatomical abnormalities [42]. Moreover, this salivary protein has been suggested to have anti-apoptotic action on taste buds [43]. Padiglia and colleagues [44] observed that different polymorphisms in the gene that codifies salivary CA-VI are related with the ability of this protein to bind zinc, and consequently with the functionality of this protein. Other proteins, such as metalloproteinases [45] and epidermal growth factor [46] have been related with taste perception, what can be done by the assistance, by these proteins, in maintaining the morphological integrity of taste buds.

The impact of salivary proteins on taste perception may also reside in their direct physico-chemical interaction with taste molecules, modifying their accessibility to receptors in taste cells. Salivary histatin 5 was reported to bind quinine and, for that reason, individuals with higher amounts of these proteins present lower sensitivity for quinine bitter taste [47]. The amino acids arginine and lysine were reported to interact with the bitter compound PROP, changing its perception [48].

Salivary proteomic studies allowed to identify a number of salivary proteins related to taste sensitivity. Cystatins [16,24], CA-VI [24] and PRPs [17] are proteins present in different amounts in individuals with different levels of bitter taste response. Whereas these proteins interact with taste molecules or have only indirect association with taste perception remains to be elucidated. One suggestion that emerged from our latest studies is that the charge of the protein may be relevant for taste perception. We did find that, more than individual proteins, were the protein forms with isoelectric point (pI) lower than salivary pH that related with PROP bitter taste responsiveness [24]. But this needs to be explored in future studies.

The influence of saliva in sweet taste perception has been less explored, comparatively to bitter taste. Our recent study (Rodrigues et al., Salivary proteome and glucose levels are related with sweet taste sensitivity in young adults, *submitted*), comparing saliva composition among young adults with different sensitivities for sucrose sweetness, presented evidences that some salivary proteins may be related with sweet taste sensitivity. These are the cases of cystatins, CA-VI and salivary α-amylase. Salivary α-amylase plays its major role in the initial digestion process by hydrolyzing α-1,4 glycosidic linkage between glucose units in the starch polysaccharide chain. The possible involvement of this protein in the perception of sensory properties of foods has already been suggested by several authors, namely by the influence on the perceived viscosity of starch [49] and the texture of semi-solid foods [50]. In the study of Rodrigues and colleagues (Rodrigues et al., Salivary proteome and glucose levels are related with sweet taste sensitivity in young adults, *submitted*), it was in individuals with higher amounts of salivary α-amylase that sucrose was perceived as less sweet. Although this has been observed to depend on sex, such negative relationship between sweetness perception and salivary α-amylase levels can be hypothesized as a result of higher constant amounts of taste substances in the oral cavity of individuals with higher amounts of this salivary protein. Salivary α-amylase has been associated with food intake and body weight in other studies: rodents with higher susceptibility for obesity

development, when subjected to high-fat diet, presented higher levels of this salivary protein, comparatively to obesity resistant animals [51]; salivary α-amylase was observed to be increased in saliva from obese women, comparatively to regular-weight pairs [52].

4. Implications of oral food perception in healthy food design and choices

Several studies in human nutrition have suggested that the PROP phenotype may serve as a general marker for oral sensations and food preferences, thus influencing dietary behavior and nutritional status [53]. Individual differences in the ability to discriminate and perceive bitter taste has aroused interest in assessing the relationship between this ability and food choices. Although some studies did not observe differences between sensitive and low sensitive individuals in food consumption, neither in adults [54] nor in children [55], others have proposed that PROP supertasters have higher sensitivity to various oral stimuli, compared to non-tasters, including bitter-tasting compounds such as dark chocolate, coffee, soy-based products and green tea [56], sweet foods, oral cavity irritants and high-fat foods [57]. Other studies did show that individuals who perceive PROP with high intensity have decreased acceptance of cruciferous vegetables, bitter fruits, spicy foods, and alcoholic beverages [58,59]. Children low sensitive to PROP bitter taste presented high intake of bitter-tasting vegetables [60], whereas children sensitive to this compound had less acceptance of foods such as spinach [61] and broccoli [62].

Given the nutritional importance of dietary lipids, the relationships between PROP status and fat perception and liking have been widely investigated. Most studies reported that PROP non-tasters had low ability to distinguish fat content and creaminess in certain fatty foods. Besides, PROP non-tasters showed higher preferences for dietary fat (such as full-fat milk, high-fat salad dressings and sweet-fat dairy mixtures) and consumed more servings of discretionary fats and high-energy foods per day than did tasters (reviewed in [53]).

4.1 Relationship between body mass index (BMI) and gustatory sensitivity

The influence of oral food perception in Body Mass Index (BMI) is naturally hypothesized due to the influence that parameters such perception has in food choices, as it has been mentioned so far. As such, studies aimed to understand the factors that influence and determine obesity have related taste perception with BMI. Most of the studies emphasize not only the relationship between sweet taste perception and obesity, but also the relationship between the perception of bitter and other tastes and this condition. Several studies suggested that obese individuals perceive sweet taste with lower intensity than normal-weight individuals and show higher preference for it [63]. However, this relationship is not consensual and other authors have not observed differences in the perception of sweet taste in individuals with different BMI [64]. Joseph and colleagues [65], in a study with children, did not observe relationship between BMI and sucrose detection thresholds, however, the authors did observe that children who presented higher weight and waist width had lower sucrose thresholds, i.e., perceived sweetness at lower concentration of tastant.

The relationship between oral sensorial perception and BMI had also been reported for fat. It was stated that obese women, despite preferring foods low in sweetness, prefer higher fat levels, comparatively to normal weight women [66].

Concerning bitter taste, and similarly to the other tastes, the way its perception level influences food choices and BMI is not consensual. Tepper and Ullrich [67] observed an inverse association between PROP phenotype and the BMI, in which adult individuals who were less sensitive to this compound had a higher BMI. On the other hand, the same group of authors [68], showed that girls sensitive to PROP had a higher BMI percentile.

4.2 Oral perception and foods design

Deep knowledge about the inter-relation between oral food perception and saliva may be used for design of healthy foods that may be appreciated by consumers. Food healthiness is one of the main driver in the creation and marketing of new food products [69–71]. The introduction of new ingredients with potential health benefits is a strategy for increasing nutritional value of foods [72]. Also the production processes used for new healthy products, which may optimise the amount of particular nutrients and to decrease the formation of potentially harmful compounds is an important strategy, for health promotion. Nevertheless, the achievement of such nutritional characteristics is many times at expense of food palatability. This, in turn, results in poor acceptability. Moreover, foods targeted at specific population groups as children [73,74] elderly or athletes [71] and intended for the prevention of specific pathological conditions [70,72] or with specific dietary needs [75] can be designed.

As such, the understanding of the factors influencing oral food perception is essential for the design of new food products, with optimised food structures and flavours, without compromising sensorial attributes that enhance the physiological regulatory mechanisms controlling appetite and energy intake having obvious benefits for weight management and help to achieve a balanced diet [76,77].

Different strategies have been used in the design of healthy foods. For example increasing the protein content of designed foods can be used as a strategy for enhancing that food satiating ability [78]. However, this strategy may affect food palatability, contributing with increased astringency or an inhomogeneous texture. As it was referred in previous

points, saliva may influence such sensorial attributes and from this point of view the evaluation of saliva composition may be useful for a better understanding and for acting at the level of changing the dynamics of in-mouth lubrication and the physical mechanisms underlying texture and mouthfeel perception [78].

Besides taste, food texture plays a key role in satiety [76]. The understanding of oral food processing in relation to the microstructure of the foods and its breakdown can lead to the development of several approaches to reduce the salt and sugar content of semi- and soft-solid foods without altering sensorial characteristics [79]. Textured foods require mastication which will slow the rate of consumption and will enhance the time of orosensory exposure [76]. During consumption the texture of foods changes continuously by chewing, shearing, mixing, heating or cooling and salivation [79], influencing perception.

The usefulness of a deep knowledge about the role of saliva in food sensory perception can be also illustrated to design healthy foods for elderly. Ageing modifies various aspects of oral physiology such as dental status, bite force, muscle fatigue and saliva composition [80] and production [81]. On the other hand a decrease in salivary flow (hyposalivation), leading to a complaint of dry mouth (xerostomia), mainly as a consequence of systemic diseases and medications is common in old people [82]. These two aspects lead to the loss of sensory abilities, resulting in changed perception of food. In turn, this may have the consequence of decreasing the pleasure felt when eating, resulting in less acceptance of eating in a healthy way. As such, old persons need foods that require little or no chewing, that are easily swallowed and that have attractive sensory characteristics [83]. Additionally, the information on optimal volume of bolus and saliva secretion for swallowing should be considered when manipulating the rheological properties of food for elderly (for detail, see [84]).

5. Conclusions

In conclusion, the influence of saliva in oral food perception and the consequent relationship between inter-individual differences in the composition of this fluid and inter-individual differences in sensorial perception can be relevant in food science. As such, it is important to start including the knowledge about this oral fluid in the strategies adopted for food design and for promoting healthy dietary habits, which will have obvious benefits for food industry. This knowledge can also be useful for this sector at the level of sensorial panel training and sensorial evaluation for new food products development.

Acknowledgements This article was funded by FCT-Science and Technology Foundation as part of Project UID/AGR/00115/2013. Funding was additionally provided by the FCT-Portuguese Science Foundation: research contract IF/01778/2013-Elsa Lamy, while FCT was not involved in carrying out this study or submitting it for publication.

References

[1] Contento IR. Determinants of Food Choice and Dietary Change: Implications For Nutrition Education. In Nutrition Education, Third Edition, Jones & Bartlett Learning, Burlington, MA, USA 2016. pp.30-58. ISBN-13: 9781284078008.

[2] Köster EP. Diversity in the determinants of food choice: A psychological perspective. Food Qual Prefer 2009. doi:10.1016/j.foodqual.2007.11.002.

[3] Paasovaara R, Luomala HT, Pohjanheimo T, Sandell M. Understanding consumers' brand-induced food taste perception: A comparison of "brand familiarity" - and "consumer value-brand symbolism (in)congruity" - accounts. J Consum Behav 2012. doi:10.1002/cb.356.

[4] Spence C. Eating with our ears: assessing the importance of the sounds of consumption on our perception and enjoyment of multisensory flavour experiences. Flavour 2015. doi:10.1186/2044-7248-4-3.

[5] Lamy E, Pinheiro C, Rodrigues L, Capela e Silva F, Lopes O, Tavares S, Gaspar R. Determinants of tannin-rich food and beverage consumption: Oral perception vs. psychosocial aspects. In: Combs CA (Ed.), Tannins: Biochemistry, Food Sources and Nutritional Properties. Nova Science Publishers 2016. pp.29-58. ISBN: 978-1-63484-150-4.

[6] Small DM, Prescott J. Odor/taste integration and the perception of flavor. Exp Brain Res 2005. doi:10.1007/s00221-005-2376-9.

[7] Besnard P, Passilly-Degrace P, Khan NA. Taste of Fat: A Sixth Taste Modality? Physiol Rev 2016. doi:10.1152/physrev.00002.2015.

[8] Lapis TJ, Penner MH, Lim J. Humans can taste glucose oligomers independent of the hT1R2/hT1R3 sweet taste receptor. Chem Senses 2016. doi:10.1093/chemse/bjw088.

[9] Chandrashekar J, Mueller KL, Hoon MA, Adler E, Feng L, Guo W, Zuker CS, Ryba NJ. T2Rs function as bitter taste receptors. Cell 2000. doi:10.1016/S0092-8674(00)80706-0.

[10] Behrens M, Meyerhof W. G Protein-Coupled Taste Receptors. Chemosensory Transduction. *The Detection of Odors, Tastes, and Other Chemostimuli* 2016. doi:10.1016/B978-0-12-801694-7.00013-5.

[11] Roper SD. Taste buds as peripheral chemosensory processors. Sem in Cell Dev Biol 2013. doi:10.1016/j.semcdb.2012.12.002.

[12] Tepper BJ. 6- n -Propylthiouracil: A Genetic Marker for Taste, with Implications for Food Preference and Dietary Habits. Am J Hum Genet 1998. doi:10.1086/302124.

[13] Wooding S, Kim U-K, Bamshad MJ, Larsen J, Jorde LB, Drayna D. Natural selection and molecular evolution in PTC, a bitter-taste receptor gene. Am J Hum Genet 2004. doi:10.1086/383092.

[14] Miller IJ, Reedy FE. Variations in human taste bud density and taste intensity perception. Physiol Behav 1990. doi:10.1016/0031-9384(90)90374-D.
[15] Ahrens W, on behalf of the IDEFICS consortium. Sensory taste preferences and taste sensitivity and the association of unhealthy food patterns with overweight and obesity in primary school children in Europe—a synthesis of data from the IDEFICS study. Flavour 2015. doi:10.1186/2044-7248-4-8.
[16] Dsamou M, Palicki O, Septier C, Chabanet C, Lucchi G, Ducoroy P, Chagnon MC, Morzel M. Salivary protein profiles and sensitivity to the bitter taste of caffeine. Chem Senses 2012. doi:10.1093/chemse/bjr070.
[17] Cabras T, Melis M, Castagnola M, Padiglia A, Tepper BJ, Messana I, Tomassini Barbarossa I. Responsiveness to 6-n-propylthiouracil (PROP) is associated with salivary levels of two specific basic proline-rich proteins in humans. PLoS One 2012. doi:10.1371/journal.pone.0030962.
[18] Morzel M, Chabanet C, Schwartz C, Lucchi G, Ducoroy P, Nicklaus S. Salivary protein profiles are linked to bitter taste acceptance in infants. Eur J Pediatr 2014. doi:10.1007/s00431-013-2216-z.
[19] Bufe B, Breslin PAS, Kuhn C, Reed DR, Tharp CD, Slack JP, Kim UK, Drayna D, Meyerhof W. The molecular basis of individual differences in phenylthiocarbamide and propylthiouracil bitterness perception. Curr Biol 2005. doi:10.1016/j.cub.2005.01.047.
[20] Dias AG, Eny KM, Cockburn M, Chiu W, Nielsen DE, Duizer L, El-Sohemy A. Variation in the TAS1R2 Gene, Sweet Taste Perception and Intake of Sugars. J Nutrigenet Nutrigenomics 2015. doi:10.1159/000430886.
[21] Dias AG, Rousseau D, Duizer L, Cockburn M, Chiu W, Nielsen D, El-Sohemy A. Genetic variation in putative salt taste receptors and salt taste perception in humans. Chem Senses 2013. doi:10.1093/chemse/bjs090.
[22] Chamoun E, Mutch DM, Allen-Vercoe E, Buchholz AC, Duncan AM, Spriet LL, Haines J, Ma DWL, & on behalf of the Guelph Family Health Study. A review of the associations between single nucleotide polymorphisms in taste receptors, eating behaviours, and health. Crit Rev Food Sci Nutr 2016. doi:10.1080/10408398.2016.1152229.
[23] Genick UK, Kutalik Z, Ledda M, Destito MCS, Souza MM, Cirillo CA, Godinot N, Martin N, Morya E, Sameshima K, Bergmann S, le Coutre J. Sensitivity of genome-wide-association signals to phenotyping strategy: the PROP-TAS2R38 taste association as a benchmark. PLoS One 2011. doi:10.1371/journal.pone.0027745.
[24] Rodrigues L, da Costa G, Cordeiro C, Pinheiro CC, Amado F, Lamy E. Relationship between saliva protein composition and 6-n -Propylthiouracil bitter taste responsiveness in young adults. J Sens Stud 2017. doi:10.1111/joss.12275.
[25] Humphrey SP, Williamson RT. A review of saliva: normal composition, flow, and function. J Prosthet Dent 2001. doi:10.1067/mpr.2001.113778.
[26] Matsuo R. Role of saliva in the maintenance of taste sensitivity. Crit Rev Oral Biol Med 2000. doi: 10.1177/10454411000110020501.
[27] Carpenter GH. The secretion, components, and properties of saliva. Annu Rev Food Sci Technol 2013. doi:10.1146/annurev-food-030212-182700.
[28] Schipper RG, Silletti E, Vingerhoeds MH. Saliva as research material: Biochemical, physicochemical and practical aspects. Arch Oral Biol 2007. doi:10.1016/j.archoralbio.2007.06.009.
[29] Salles C, Chagnon M-C, Feron G, Guichard E, Laboure H, Morzel M, Semon E, Tarrega A, Yven C. In-mouth mechanisms leading to flavor release and perception. Crit Rev Food Sci Nutr 2011. doi:10.1080/10408390903044693.
[30] Lamy E, da Costa G, Santos R, Capela e Silva F, Potes J, Pereira A, Coelho AV, Sales Baptista E. Sheep and goat saliva proteome analysis: a useful tool for ingestive behavior research? Physiol Behav 2009. doi:10.1016/j.physbeh.2009.07.002.
[31] Lamy E, Graça G, da Costa G, Capela e Silva F, Baptista ES, Coelho AV. Changes in mouse whole saliva soluble proteome induced by tannin-enriched diet. Proteome Sci 2010. doi:10.1186/1477-5956-8-65.
[32] Lamy E, da Costa G, Santos R, Capela e Silva F, Potes J, Pereira A, Coelho AV, Baptista ES. Effect of condensed tannin ingestion in sheep and goat parotid saliva proteome. J Anim Physiol Anim Nutr (Berl) 2011. doi:10.1111/j.1439-0396.2010.01055.x.
[33] Williamson MP. The structure and function of proline-rich regions in proteins. Biochem J 1994. doi: 10.1042/bj2970249.
[34] Yan Q, Bennick A. Identification of histatins as tannin-binding proteins in human saliva. Biochem J 1995. doi:10.1042/bj3110341.
[35] Soares S, Mateus N, de Freitas V. Interaction of different classes of salivary proteins with food tannins. Food Res Int 2012. doi:10.1016/j.foodres.2012.09.008.
[36] Dinnella C, Recchia A, Vincenzi S, Tuorila H, Monteleone E. Temporary modification of salivary protein profile and individual responses to repeated phenolic astringent stimuli. Chem Senses 2010. doi:10.1093/chemse/bjp084.
[37] Canon F, Giuliani A, Paté F, Sarni-Manchado P. Ability of a salivary intrinsically unstructured protein to bind different tannin targets revealed by mass spectrometry. Anal Bioanal Chem 2010. doi:10.1007/s00216-010-3997-9.
[38] McColl J, Horvath R, Aref A, Larcombe L, Chianella I, Morgan S, Yakubov GE, Ramsden JJ. Polyphenol control of cell spreading on glycoprotein substrata. J Biomater Sci Polym Ed 2009. doi:10.1163/156856209X427023.
[39] Davies HS, Pudney PDA, Georgiades P, Waigh TA, Hodson NW, Ridley CE, Blanch EW, Thornton DJ. Reorganisation of the salivary mucin network by dietary components: insights from green tea polyphenols. PLoS One 2014. doi:10.1371/journal.pone.0108372.
[40] Spielman AI, D'Abundo S, Field RB, Schmale H. Protein analysis of human von Ebner saliva and a method for its collection from the foliate papillae. J Dent Res 1993. doi:10.1177/00220345930720091301.
[41] Schmale H, Holtgreve-Grez H, Christiansen H. Possible role for salivary gland protein in taste reception indicated by homology to lipophilic-ligand carrier proteins. Nature 1990. doi:10.1038/343366a0.
[42] Henkin RI, Martin BM, Agarwal RP. Efficacy of exogenous oral zinc in treatment of patients with carbonic anhydrase VI deficiency. Am J Med Sci 1999. doi:10.1097/00000441-199912000-00006.
[43] Leinonen J, Parkkila S, Kaunisto K, Koivunen P, Rajaniemi H. Secretion of carbonic anhydrase isoenzyme VI (CA VI) from human and rat lingual serous von Ebner's glands. J Histochem Cytochem 2001. doi:10.1177/002215540104900513.
[44] Padiglia A, Zonza A, Atzori E, Chillotti C, Calò C, Tepper BJ, Barbarossa IT. Sensitivity to 6-n-propylthiouracil is associated

with gustin (carbonic anhydrase VI) gene polymorphism, salivary zinc, and body mass index in humans. Am J Clin Nutr 2010. doi:10.3945/ajcn.2010.29418.
[45] Watanabe M, Asatsuma M, Ikui A, Ikeda M, Yamada Y, Nomura S, Igarashi A. Measurements of several metallic elements and matrix metalloproteinases (MMPs) in saliva from patients with taste disorder. Chem Senses 2005. doi:10.1093/chemse/bji007.
[46] Morris-Wiman J, Sego R, Brinkley L, Dolce C. The effects of sialoadenectomy and exogenous EGF on taste bud morphology and maintenance. Chem Senses 2000. doi:10.1093/chemse/25.1.9.
[47] Wada H, Yamamori T, Shimazaki N, Ishibashi K, Seino K. Function of salivary histatin 5 for bitter substances. Conference Paper, IADR 88th General Session and Exhibition, Barcelona, 2010.
[48] Melis M, Aragoni MC, Arca M, Cabras T, Caltagirone C, Castagnola M, Crnjar R, Messana I, Tepper BJ, Barbarossa IT. Marked increase in PROP taste responsiveness following oral supplementation with selected salivary proteins or their related free amino acids. PLoS One 2013. doi:10.1371/journal.pone.0059810.
[49] Mandel AL, Peyrot des Gachons C, Plank KL, Alarcon S, Breslin PAS. Individual differences in AMY1 gene copy number, salivary α-amylase levels, and the perception of oral starch. PLoS One 2010. doi:10.1371/journal.pone.0013352.
[50] Engelen L, van den Keybus PAM, de Wijk RA, Veerman ECI, Amerongen AVN, Bosman F, Prinz JF, van der Bilt A. The effect of saliva composition on texture perception of semi-solids. Arch Oral Biol 2007. doi:10.1016/j.archoralbio.2006.11.007.
[51] Rodrigues L, Mouta R, Costa AR, Pereira A, Capela e Silva F, Amado F, Antunes CM, Lamy E. Effects of high fat diet on salivary α-amylase, serum parameters and food consumption in rats. Arch Oral Biol 2015. doi:10.1016/j.archoralbio.2015.02.015.
[52] Lamy E, Simões C, Rodrigues L, Costa AR, Vitorino R, Amado F, Antunes C, do Carmo I. Changes in the salivary protein profile of morbidly obese women either previously subjected to bariatric surgery or not. J Physiol Biochem 2015. doi:10.1007/s13105-015-0434-8.
[53] Tepper BJ, Banni S, Melis M, Crnjar R, Tomassini Barbarossa I. Genetic sensitivity to the bitter taste of 6-n-propylthiouracil (PROP) and its association with physiological mechanisms controlling body mass index (BMI). Nutrients 2014. doi:10.3390/nu6093363.
[54] Martinez-Ruiz N del R, Wall-Medrano A, Jimenez-Castro JA, Lopez-Diaz JA, Angulo-Guerrero O. Relación entre el fenotipo PROP, el índice de masa corporal, la circunferencia de cintura, la grasa corporal total y el consumo dietario. Nutr Hosp 2014. doi:10.3305/nh.2014.29.1.6982.
[55] Vance SJ, McDonald RE, Cooper A, Smith BO, Kennedy MW. The structure of latherin, a surfactant allergen protein from horse sweat and saliva. J R Soc Interface 2013. doi:10.1098/rsif.2013.0453.
[56] Gayathri Devi A, Henderson SA, Drewnowski A. Sensory acceptance of Japanese green tea and soy products is linked to genetic sensitivity to 6-n-propylthiouracil. Nutr Cancer 1997. doi:10.1080/01635589709514616.
[57] Hayes JE, Duffy VB. Revisiting sugar-fat mixtures: Sweetness and creaminess vary with phenotypic markers of oral sensation. Chem Senses 2007. doi:10.1093/chemse/bjl050.
[58] Drewnowski A, Gomez-Carneros C. Bitter taste, phytonutrients, and the consumer: a review. Am J Clin Nutr 2000. PMID:11101467.
[59] Sandell M, Hoppu U, Mikkilä V, Mononen N, Kähönen M, Männistö S, Rönnemaa T, Viikari J, Lehtimäki T, Raitakari OT. Genetic variation in the hTAS2R38 taste receptor and food consumption among Finnish adults. Genes Nutr 2014. doi: 10.1007/s12263-014-0433-3.
[60] Bell KI, Tepper BJ. Short-term vegetable intake by young children classified by 6-n-propylthoiuracil bitter-taste phenotype. Am J Clin Nutr 2006. PMID:16825702.
[61] Turnbull B, Matisoo-Smith E. Taste sensitivity to 6-n-propylthiouracil predicts acceptance of bitter-tasting spinach in 3-6-y-old children. Am J Clin Nutr 2002. PMID:12399285.
[62] Keller KL, Steinmann L, Nurse RJ, Tepper BJ. Genetic taste sensitivity to 6-n-propylthiouracil influences food preference and reported intake in preschool children. Appetite 2002. doi:10.1006/appe.2001.0441.
[63] Bartoshuk LM, Duffy VB, Hayes JE, Moskowitz HR, Snyder DJ. Psychophysics of sweet and fat perception in obesity: problems, solutions and new perspectives. Philos Trans R Soc Lond B Biol Sci 2006. doi:10.1098/rstb.2006.1853.
[64] Anderson GH, Navia JM, Hill JO, Miller B, Prentice A. Sugars, sweetness, and food intake. Am J Clin Nutr 1995. PMID:7598077.
[65] Joseph PV, Reed DR, Mennella JA. Individual differences among children in sucrose detection thresholds: Relationship with age, gender, and bitter taste genotype. Nurs Res 2016. doi:10.1097/NNR.0000000000000138.
[66] Drewnowski A, Brunzell JD, Sande K, Iverius PH, Greenwood MRC. Sweet tooth reconsidered: Taste responsiveness in human obesity. Physiol Behav 1985. doi:10.1016/0031-9384(85)90150-7.
[67] Tepper BJ, Ullrich NV. Influence of genetic taste sensitivity to 6-n-propylthiouracil (PROP), dietary restraint and disinhibition on body mass index in middle-aged women. Physiol Behav 2002. doi: 10.1016/S0031-9384(01)00664-3.
[68] Keller KL, Tepper BJ. Inherited taste sensitivity to 6-n-propylthiouracil in diet and body weight in children. Obes Res 2004. doi:10.1038/oby.2004.110.
[69] Palzer S. Food structures for nutrition, health and wellness. Trends Food Sci Technol 2009. doi:10.1016/j.tifs.2009.02.005.
[70] Zúñiga RN, Troncoso E. Improving Nutrition Through the Design of Food Matrices. In Valdez B (Ed.), Scientific, Health and Social Aspects of the Food Industry. InTech 2012. pp.295-320. doi:10.5772/33504.
[71] Norton JE, Wallis GA, Spyropoulos F, Lillford PJ, Norton IT. Designing food structures for nutrition and health benefits. Ann Rev Food Sci Technol 2014. doi:10.1146/annurev-food-030713-092315.
[72] Rajasekaran A, Kalaivani M. Designer foods and their benefits: A review. J Food Sci Technol 2013. doi:10.1007/s13197-012-0726-8.
[73] Guinard JX. Sensory and consumer testing with children. Trends Food Sci Technol 2000. doi:10.1016/S0924-2244(01)00015-2.
[74] Kilcast D, Angus F. Developing Children's Food Products. Woodhead Publishing Limited, Cambridge, UK 2011. ISBN 978-1-84569-431-9
[75] Osborn S, Morley W. Developing Food Products for Consumers with Specific Dietary Needs. Woodhead Publishing Limited,

Cambridge, UK 2016. ISBN: 9780081003299.
[76] Chambers L, McCrickerd K, Yeomans MR. Optimising foods for satiety. Trends Food Sci Technol 2015. doi:10.1016/j.tifs.2014.10.007.
[77] Campbell CL, Wagoner TB, Foegeding EA. Designing foods for satiety: The roles of food structure and oral processing in satiation and satiety. Food Struct 2017. doi:10.1016/j.foostr.2016.08.002.
[78] Morell P, Chen J, Fiszman S. The role of starch and saliva in tribology studies and the sensory perception of protein-added yogurts. Food Funct 2017. doi:10.1039/C6FO00259E.
[79] Stieger M, van de Velde F. Microstructure, texture and oral processing: New ways to reduce sugar and salt in foods. Curr Opin Colloid Interface Sci 2013. doi:10.1016/j.cocis.2013.04.007.
[80] Ship JA. The influence of aging on oral health and consequences for taste and smell. Physiol Behav 1999. doi:10.1016/S0031-9384(98)00267-4.
[81] Turner MD, Ship JA. Dry mouth and its effects on the oral health of elderly people. J Am Dent Assoc 2007. doi:10.14219/jada.archive.2007.0358.
[82] Gupta A, Epstein JB, Sroussi H. Hyposalivation in elderly patients. J Can Dent Assoc 2006. PMID:17109806.
[83] Hall G, Wendin K. Sensory design of foods for the elderly. Ann Nutr Metab 2008. doi:10.1159/000115344.
[84] Nishinari K, Takemasa M, Brenner T, Su L, Fang Y, Hirashima M, Yoshimura M, Nitta Y, Moritaka H, Tomczynska-Mleko M, Mleko S, Michiwaki Y. The Food Colloid Principle in the Design of Elderly Food. J Texture Stud 2016. doi:10.1111/jtxs.12201.

Use plant based-recombinant proteins in food and human health

C.A. Oluk and **P. Cubukcu**

Ministry of Food Agriculture and Livestock, Eastern Mediterranean Agriculture Research Institute, 01321 Adana, Turkey

Recombinant protein is a manipulated form of protein, which is generated in various ways to produce large quantities of proteins, modify gene sequences and manufacture useful commercial products. The formation of recombinant protein is carried out in specialized vehicles known as vectors.

Plants are chosen because they are ideally suitable for producing recombinant proteins with many advantages over other systems. First, compared to the traditional microbial fermentation, plants are able to perform post-translational modifications, such as glycosylation and hydroxylation, which are required for biological activity of numerous mammalian proteins. Second, plants, especially crops, can produce exogenous proteins on large scale at very low cost, since plant growth, transport, and post-harvest process requirements are relatively inexpensive . Third, unlike animal or microorganism systems, plant-derived products reduce contamination risk due to human pathogens. Fourth they are easily transformed and provide a cheap source of protein. There are also drawbacks for transgenic plant production; one of the concerns is that the timescale of production is relatively long compared with plant or animal cell cultures and microorganisms

Most major groups of proteins have been produced successfully in a diverse variety of crops such as maize, rice, wheat, soybean, tomato, potato, mustard, oilseed rape, turnip, alfalfa, banana and tobacco. We will briefly review the recent developments in plant-based recombinant production technology.

Keywords: recombinant proteins; plant-derived products; protein crops

1. Plant made recombinant proteins

As a result of fast developing biotechnology during past few decades, nowadays plants can be used to produce various heterologous proteins, including pharmaceutical and industrial proteins, through recombinant DNA technology, often referred to as plant molecular farming [1]. Plant-based systems provide the spectrum of production capacity ranging from plant/algal cell bioreactor systems for lower volume, higher value product to field-grown commodity crops with potential for metric tons of recombinant protein at highly competitive costs. Because plants cannot harbor the human and animal pathogens-of-issue for mammalian cell-based production systems, they bring significant advantage in increased safety for patients[2].

Plant-made recombinant proteins can be generally categorized into three classes: therapeutic proteins, industrial proteins/enzymes and biopolymers

1.1 Therapeutic proteins

Therapeutic proteins include monoclonal antibodies (mAbs), vaccine antigens, therapeutic enzymes, blood proteins, cytokines, growth factors and growth hormones[2]. Bioactivity of these proteins often requires protein folding, disulfide bond formation, subunit assembly, proteolytic cleavage, and/or glycosylation, highlighting the ability of plants to process complex human/mammalian proteins. Plant-made antibodies have received considerable interest as they are made at much lower cost in plants than inmammalian cells without the associated risks of potentially harboring animal pathogens [3]. Plants successfully glycosylate proteins at the signature recognition motif (N-X-S/T) for N-linked glycosylation. However, subsequent processing in the Golgi to complex glycans differs from that found in mammalian cells. Thus, a notable challenge in using plants as hosts for production of glycosylated therapeutic proteins is that plantspecific xylose and α-1,3-fucose sugars may be added with a potential to alter bioactivity or immunogenicity in humans[4]

Antibodies and vaccines are the two major classes of plant-made therapeutic proteins that are under commercial development. Planet Biotechnology took the lead in developing and commercializing Plantibodies produced in tobacco; those being tested in clinical trials include CaroRX (for dental caries), DoxoRX (for side-effects of cancer therapy), RhinoRX (for Rhinovirus prophylactic) and an IgG (ICAM1) (for common cold) [5] In addition, a 2G12 IgG used as prophylactic treatment for HIV is in Phase I clinical trials by the Pharma-Planta Consortium) [6], and a plant-made scFv monoclonal antibody, used in downstream processing of a hepatitis B vaccine, has been commercialized in Cuba) [7].

1.2 Industrial enzymes

Transgenic plants provide a viable technology for producing industrial proteins, in particular enzymes, because of low cost of agricultural production, stability of protein stored in specific organs such as seeds, ease and speed of scale-up as

well as the possibility of using crude plant materials directly in industrial processes [8]. These are hydrolases, including glycosidases (e.g., cellulase, α-amylase and β-glucuronidase (GUS) and proteases (e.g., trypsin). Corn seed is regarded as an ideal platform for the production of industrial proteins/enzymes because corn has the largest annual grain yield and relatively high seed protein content (10%), thus offering the highest potential recombinant protein yields per hectare. Corn-produced enzymes such as cellulases and hemicellulases involved in biomass conversion to produce biofuels such as ethanol are currently interesting candidates for commercialization [5].

1.3 Biopolymers

Recombinant biopolymers are spider silk proteins, elastin-like polypeptides (ELPs), collagens and plant gums. The spider silk proteins (spidroins) that are modular fibrous proteins containing highly repetitive amino-acid sequences consisting largely of glycine and alanine [9].Spider silk fibers spun from these spidroins are regarded as one of nature's most extraordinary materials with exceptional flexibility, elasticity, and toughness—three times as strong as Kevlar and five times as strong as steel [10].Plants offer a more efficient and cheaper production platform than bacteria for production of recombinant spidroins. ELPs that are comprised of the repetitive pentapeptide sequence (VGVPG), which mainly serves as thermally responsive tags for the non-chromatographic purification of recombinant proteins [11]. ELP tags werefound to significantly enhance the production yield of a range of different recombinant proteins in plant leaves [12].

2. Plant expression systems

The rapid development of plant genetic engineering technologies has expanded the diversity of well-established plant based bioproduction systems for recombinant proteins [12]. The used plants have advantages and disadvantages (Table 1). Each of the systems has its own strengths and weaknesses, which are described here.

Table 1 Comparison of advantages and disadvantages associated with the three major types of plant.

	Advantages	**Disadvantages**
Seed crops	✓ Grown in open-fields, ✓ the economy of scale, ✓ established agricultures practices, ✓ Seed proteins do not degrade at ambient temperature ✓ Stable for long term storage ✓ seed has superior aspects as a mucosal delivery vehicle for oral peptide／protein therapeutics ✓ improved consistency of protein product with the use of controlled bioreactors which are less prone to biotic- and abiotic-induced variations that commonly hamper field/greenhouse grown plant-based protein production	✓ lower biomass yields ✓ They have long growth cycle ✓ Not suitable for transient expressions ✓ In the pollination period, the potential transgene could escape into the enviroment ✓ Issues of gene silencing of nuclear transgenes limit production performance
Leafy crops	✓ High biomass yield, ✓ possibility for multiple growth cycles per year, ✓ established agricultural infrastructure like seed systems ✓ less risk of pollen spreading (flowering can be prevented) ✓ low-up front capitilazation costs	✓ using transient expression ✓ applied specifically to expression of monoclonal antibodies (mAbs), vaccines, and other therapeutics products ✓ high water content, ✓ Non storage stability of harvested biomass, ✓ recombinant protein stability which does not allow decoupling of upstream and downstream processing. ✓ phenolic compounds, and chlorophyll-derived pigments that could pose difficulties during downstream processing
Aquatic plants	✓ safe ✓ fast-growing ✓ easy to grow and harvest ✓ has a high protein content ✓ offers an attractive system for oral vaccines	✓ contain small amounts of phenolics (primarily ferulic and coumaric acids), phytic acid, lipids, and lectins that may interfere with protein purification ✓ Difficulty in culture scale up ✓ Higher capital investment

The selection of the promoter is the first thing to consider before making the construct. Promoter elements can dramatically affect the level of messenger RNA and, thus, influence the accumulation of the protein[14]. To obtain high transcription levels, many constitutive promoters have been used to drive the transgene. The most widely used promoter is the cauliflower mosaic virus (CaMV) 35S promoter [15], but it is much weaker in monocotyledonous plants. In dicotyledonous plants, strong constitutive promoters, such as rice actin-1 or maize ubiquitin-1, are more frequently used for expressing foreign proteins [16].

Codon usage should be considered when a transgene is expressed in a host plant with different codon use frequency to achieve optimal expression levels. Nowadays, codon optimization is a standard practice for transgene expression. Many codon optimization programs are available on the Internet based on the analysis of sequences available for a given species[17,18].

Subcellular targeting is an alternative strategy to enhance the expression of exogenous proteins by avoiding protein degradation caused by proteases in the host plant. Recombinant proteins can be targeted to the cell wall, the vacuole, the mitochondria, and the chloroplast by fusing with different targeting signal. If a targeting signal sequence is not designed, the recombinant protein may be released in the cytoplasm where the protein could be degraded by proteases [19]. The endoplasmic reticulum (ER) is an ideal target compartment for recombinant proteins. The ER retention sequences, such as KDEL (Lys-Asp-Glu-Leu) or HDEL (His-Asp-Glu-Leu), are used to keep recombinant proteins within the endoplasmic reticulum. This strategy has significantly enhanced the expression of foreign proteins in transgenic plants[20].

Protein fusion approaches are effective for boosting the expression, increasing solubility and stability, and facilitating isolation and purification of recombinant proteins [21]. Zera is maize seed storage protein γ- zein derived proline-rich N-terminus domain, which has self-assembling and protein body formation properties [14]. Another successful fusion partner example is elastin-like polypeptides, which are artificially-designed biopolymers.

In the maize *o2* mutant, non-zein proteins have increased, while zeins are reduced significantly, resulting in improved maize seed nutritional quality [22]. In cotton, the RNAi mediated knock-down of two key fatty acid desaturase genes leads to the increase of high-oleic and high-stearic cotton seed oil, essential fatty acids for human heart health [23]. In soybean, while the expression of α and α' subunits of β-conglycinin is suppressed by sequence-mediated gene silencing in seeds, another soybean seed storage protein glycinin is increased to balance the total seed protein [24]. A strategy is raised to improve the nutritional quality or to enhance the expression of recombinant proteins in seeds, using the gene silencing mechanism to reduce a major seed protein gene [25].In *Arabidopsis thaliana*, a transgene *arcelin5-I* from *Phaseolus vulgaris* increased up to 15% of the total soluble protein of the seed when the endogenous seed storage protein 2S albumin is silenced by antisense. Two major maize seed storage proteins, α- and γ-zeins, have been silenced by antisense or RNAi methods [26]. Non-zein proteins increased in these transgenic maize lines and seed nutritional quality improved high-lysine maize [27].

2.1 Stable Seed based systems

Seeds have used many applications in the area of molecular farming. Seed provides its genotype with powerful selective advantages because of its flexibility in the face of a changing environment. They provide a plant with the ability to germinate and grow under nutrient-limited conditions because of the availability of storage products. Seeds allow germination to take place under environmental conditions, which most favour the seedling's survival[28]. Seeds are naturally designed for protein storage for long periods of time without or with very little degradation [29]. Seed dormancy is a very important property of many seeds. The ability of a seed to go into a quiescent state for long periods or until an appropriate environmental signal is detected provides a substantial survival advantage for many wild species. The stability of the recombinant protein and the dormancy of the seed also allows for a complete decoupling of the cycle of cultivation from the processing and purification of the protein. Current Good Manufacturing Practices (cGMP) manufacturing, this is extremely helpful as it permits the establishment of such concepts as a 'master seed bank' and also allows for the establishment of quality-based release criteria for the seeds as a precursor. Seeds can be stored after harvest without cooling or immediate isolation that are required for leafy tissues [28]

Proteins expressed in seeds are generally protected from proteolytic degradation, and storage upwards of three years at room temperature (longer with cold storage) resulted in minimal loss of protein activity. Seeds have a relatively lower biomass and higher cross-contamination risk by pollen drift for non-self-pollinated plants. However, considering the stability of foreign proteins, post-harvest processing, and overall cost, the seed-based platform is still more suitable for many recombinant proteins production on a large scale [30]. Localization of storage products at the cellular level is a major determinant of accumulation levels of the product as well as the potential stability of the product during prolonged periods of dormancy. For example, a recombinant protein targeted to the cytoplasm may accumulate at moderate levels, but never satisfactory levels, whereas the same protein in a configuration which targets the secretory pathway can accumulate at high levels suitable for economic production [28]. Recombinant protein accumulation in seed has been shown to mimic the abilities of naturally occurring proteins. So, for example ,in rice, the use of storage protein promoters to drive expression of genes for mammalian proteins such as human lysozyme has resulted in an average expression level of 13%–14% of total soluble protein[31]. Neither seed nor leaf extraction can be performed at a large scale under aseptic conditions, most seeds can be subjected to a surface 'sterilization' technique, which reduces bioload to industrially accepted standards for a raw material. This is difficult if not impossible to do with a large mass of leaves without damaging the mesophyll cells, which carry the bulk of the recombinant product. One of the disadvantage in seed-based expression systems is speed to proof-of-concept, or in the case of recombinant proteins required in relatively smaller volumes, the time-to-product may be preclusive. [28].

The first plant-derived commercialized product was produced in maize derived avidin and trypsin. Hovewer, oil-seeds are emerging as a promising platform for recombinant protein production due to their inherently low associated

proteolytic activity and simplified protein isolation via oil body separations. During the past decade, plants have emerged as promising biopharming systems for commercial production of pharmaceutical proteins (Table 2). SemBioSys Genetics (Calgary, Canada), which has developed the oleosin-fusion platform, in which the target recombinant protein is produced as a fusion with oleosin and accumulates in safflower oilbodies (www.sembiosys.com/). Ventria Biosciences and Meristem Therapeutics, which have invested in field-grown rice for the production of the human proteins lactoferrin and lysozyme (www.ventria.com/). ORF Genetics Ltd., based in Iceland, has targeted barley grain as the expression site for a number of human cytokines and growth factors (www.orfgenetics.com/).

Table 2 Plant-derived pharmaceutical products in clinical development manufactured.

Product	**Platform-plant**	**Comments**
Escherichia coli heat-labile enterotoxin (LT-B)	Transgenic potato tubers	[32]
Norwalk virus coat protein		[33]
Norwalk virus capsid protein		[34]
Rabbit haemorrhagic disease virus (RHDV) VP60		[35]
HBsAg		[36,37]
Combination cholera⁄ E. coli⁄ rotavirusvaccine		[38]
Human papilloma virus E7 and L1 proteins		[39,40,41]
Newcastle disease virus envelope proteins		[42]
ZMapp	Tobacco leaves	[43]
Immuno adhesin (DPP4-Fc)	Transgenic tobacco	[44]
Hepatitis B surface antigen (HBsAg)		[45]
Human growth hormone	Tobacco and sunflower	[46]
Taliglucerase alfa; Recombinantglucocerebrosidase (prGCD)	Carrot cellculture	[47]
Therapeuticenzym (Glucocerebrosidase (UPLYSO)		[48,49]
Human Vascular endothelial growth factor (VEGF)	Barley	[50,51]
Trypsin	Transgenic maize	[52]
Soybean derived monoclonal antibodies(mAbs)	Soybean	[53]
Human acidic fibroblast growth factor 1 (FGF-1)	Salviamiltiorrhiza	[54]
Therapeutic protein (Apo-A1 ^{Milano})	Saffflower	[55]
Antibody(Anti-CD20 mAb (BLX-301)	Lemna (Duckweed)	[56]
Human serum albumin	Flax	[57]
Lactoferrin	Transgenic rice	[58,59]
Collagen	Transgenic maize	[60]
Therapeutic enzyme		[61]
Human intrinsic factor	Transgenic Arobidopsis	[62,63]
Pandemic and seasonal influenza vaccines	N. benthamiana	[64]
Hepatitis B surface antigen	Transgenic potato Transgenic lettuce	[65,66]
Rabies glycoprotein	Viral vectors in spinach	[67]
Various anti-idiotype IgG antibodies clinical trial NCT01022255	N. benthamiana	[68,69]
hGAD67/ 65 mut	Arabidopsis Thalina Petunia	[70]
2 G12	Maize, Arabidopsis Thalina	[71,72,73]
NVCP	Lettuce	[74]
	Potato	[75]
	Tomato	
hGAD65	Carrot	[76]

2.2 Stable Leaf based systems

Tobacco is the leading leaf-based protein expression system for commercial products [1]. The first commercial products were tobacco-derived secretory IgA. Tobacco is genetically well studied and easily manipulated, is classified as a non-food/non-feed crop, produces high biomass (upwards of 300 tons per acre) and is one of the best studied platforms to date for expressing recombinant biopharmaceuticals. [77]. Many other leafy crops have been used for stable expression of proteins including lettuce, alfalfa and clover. The use of crops like alfalfa has additional benefits; it is a perennial that fixes nitrogen and displays notable homogeneity of N-glycosylated recombinant proteins[78].The process of extracting

recombinant proteins from leafy plants does not require additional operations, such as seed grinding, soaking, and de-oiling [30], but leafy plants have high water content and low storage stability [3]. Leaf-based systems are able to produce a high yield of recombinant proteins, this yield may be offset by an increased instability of expressed proteins in metabolically active tissues, such as leaves [3].

Both nuclear and plastid integration have been used when expressing recombinant proteins in leaf tissue. The choice of gene integration within the plant cell is generally dictated by the post-translational requirements of the target protein. For glycosylated proteins, nuclear integration of the transgene is needed to enable proper processing of the protein in the endomembrane system [4].There are many cases of recombinant proteins in leaves having associated stability and accumulation issues. Product yields in field-grown materials can be highly variable due to environmental impacts (both biotic and abiotic stresses) leading to increased consideration of growth in more controlled conditions (e.g., under plastic or in greenhouses), especially for pharmaceutical applications [13]. For example, algae possess a number of advantages over transgenic plant systems for the production of recombinant proteins. They can be grown in contained bioreactors, reducing the risk of contamination of the production system by airborne contaminants, and also protecting the environment from any potential flow of transgenes into the surrounding ecosystem. Growth in containment also greatly reduces the potential for loss of the crop due to predation or pathogen attack[79]. Robert [80], assessed the potential of methyl jasmonate (MeJA) as a generic trigger of recombinant protein enrichment in Nicotiana benthamiana leaves before harvesting. They found that overall the ability of MeJA trigger ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCO) depletion and recombinant protein enrichment in N. benthamiana leaves.

2.3 Transient Expression System

Transient expression systems, mediated by recombinant viral or plant binary vectors, are being increasingly used for the expression of biopharmaceuticals in plants due to speed, high protein yields, and regulatory considerations. During this process, foreign genes are typically introduced into leaf tissue of intact plants (generally non-transgenic) by vacuum infiltration of engineered Agrobacterium (containing gene(s) of interest within T-DNA with or without additional virus-derived components). Recombinant protein production (based on extrachromosomal gene expression within the plant cell) is initiated in the leaves within 24 h and continues for several days (Agro-mediated) to several weeks (viral mediated) depending on vector and protein. While chromosomal integration is possible, it occurs at considerably lower frequency in comparison to the number of cells transiently expressing the desired gene [81].

There are two basic approaches for plant transient expression based on the mechanism by which the transgene is transferred into the plant cell: viral-mediated or plant binary vector-based [82]. The accumulation of recombinant proteins in plants occurs within a shorter time, typically 2–4 days post-infiltration, with recoveries typically ranging from ~0.1 to 180 μg/g fresh leaf depending on the gene of interest by conventional" plant expression vectors, [83]. Plant viral vectors also use the same Agro-infiltration system to introduce DNAcopies into the plant cell. This transient expression process generally produces higher levels of recombinant protein with yields reported to be as high as 5 mg/g of fresh leaf for GFP (green fluorescence protein gene) [84. However, recombinant protein expression by this process takes up to 14 days, which can present issues for proteins prone to protease degradation and instability. Recombinant protein production using transient expression is now being mobilized to large scale by industry such as pharmaceuticals and vaccines. The production of large quantities of recombinant protein offered by transient plant expression systems, coupled with use of current technology to increase yields and many technical promising solutions seem to favorably compare with mammalian or insect cellbased systems in quality, cost, and scale. [13].

The production of vaccines using a transient plant expression system has been developed rapidly, somewhat due to the recent outbreak of avian and swine flu, which led to the development of seasonal and pandemic influenza vaccines.

2.4 In Vitro Culture System

In vitro culture systems are characterized by the fact that plant biomass is cultured in confined bioreactors under sterile conditions for large-scale production of recombinant proteins.

Like microorganisms, undifferentiated clusters of plant cells (callus) can be dispersed and propagated in a liquid medium to generate stable cell suspension cultures, which can retain the same production capacity as whole plants. Increased concerns about regulatory compliance and product safety of mammalian systems recently have renewed interest in plant cell cultures as an alternative production platform for complex pharmaceutical proteins. These systems provide cGMP-compatible production environments more acceptable to the established pharmaceutical industry with added benefits of complex protein processing compared to bacteria and yeasts, and increase safety compared tomammalian cell systemswhich can harbor human pathogens. [13].

Hairy roots are generated by infection of plants with Agrobacterium rhizogenes that harbors a large root-inducing plasmid [85]. Hairy roots can be axenically cultured in a controlled environment suitable for the production of high-value pharmaceutical proteins under cGMP requirements. In addition, the possible extracellular secretion of expressed proteins from cultured hairy roots, or rhizosecretion The advantages of rapidly growing hairy roots over suspension cells include long-term genotype and phenotype stability, efficient productivity and the ability of hairy roots to grow on

hormone-free medium. [13].The challenges for large scale culture of hairy roots because they have a tangled, filamentousmorphology and often possess unique physiological characteristics.

3. Strategies of recombinant proteins

There are several strategies for the primary recovery and purification of recombinant proteins from seed, leafy, and bioreactor-based platforms. They are upstream processing and downstream processing. These strategies will be briefly reviewed below:

3.1 Downstream processing

Downstream processing efficiency is influenced by the recombinant protein concentration, the complexity of the plant extracts or cell-free culture media, and required final product purity. Downstream processing can be divided into two phases: primary recovery and purification [86].

The primary recovery steps for leafy- and seed-based production systems consist of product release from the biomass by homogenization or aqueous extraction; solid–liquid separation; and conditioning, pretreatment, and clarification. Some primary recovery unit operations, such as centrifugation, cross-flow filtration, and dead-end filtration, are multi-functional, and their strategic applications are defined by the extract composition, particle size, extract properties, and product stability [1].

Fractionation uses established processing methods, such as dry milling, dry fractionation, and wet milling, to reduce total processing volume and solids content, to enrich the recombinant protein, and to generate co-product revenues for seed-based systems [87].

The protein release/extraction process is a critical recovery step because it dictates the total extract volume, recombinant protein concentration and purity, and the type and quantity of impurities that have to be removed during purification [88]. Ground seed is extracted with low-shear mixers using 4 to 5 L of buffer per kg dry seed. Extraction of oil bodies for oleosin-fusion technology requires much greater water-to-tissue ratios [89]. The ratio of recombinant protein to native protein is another critical factor for reducing the purification burden that can be easily manipulated by adjusting the extraction buffer pH and ionic strength and plant-tissue-to-buffer ratio[90]. For example, extraction of corn seed with pH 3 buffer instead of pH 9 bufferresulted in an eight-fold decrease in native protein concentration and a two-fold decrease for the same pH range for tobacco leaves [91]. Low pH extraction (pH 3) allowed easier purification of collagen and aprotinin from transgenic corn extracts [92].

Centrifugation is a common method for solids removal and/or clarification of plant extracts and homogenates because continuous or semi-continuous processing is feasible, the process is easy to scale up, and can handle a variety of solid–liquid suspensions.

Plant extracts and plant cell homogenates have a unique composition of native proteins, cell metabolites, and process-derived impurities, and the presence of these potentially harmful impurities may require distinct handling or conditioning of the extract. The purpose of conditioning is to maximize product binding by capture chromatography, reduce binding of plant components to the recombinant protein, and stabilize extracts for purification. Traditional conditioning methods, also referred to as pretreatment methods, for any protein production platforms include adjustment of extract or media pH, ionic strength, buffer composition, and volume reduction by cross-flow filtration. [1]. Several approaches have been implemented specifically for pretreatment of plant extracts including aqueous two-phase partitioning, adsorption, precipitation, and membrane filtration.

The purification phase usually starts with a capture step to concentrate the recombinant protein and most importantly, remove critical plant impurities that would be detrimental to protein yield, quality, and/or purification efficiency [93]. Purification of plant-derived proteins, similarly to other protein production systems, is dependent on protein properties and host cell impurities, and utilizes similar strategies for purification development as other heterologous expression systems. There are very few studies reporting purification processes for plant-derived recombinant proteins that have reached clinical trials or been scaled-up to commercial production. The reason for this is twofold. First, relatively few companies have reached later stages of clinical trials (Phases II and III) to have established robust, at-scale downstream processes and, second, newly developed processes are often deemed proprietary [28].

Capture chromatography resins have two primary functions, concentration and partial purification, and should be inexpensive, resistant to chemicals needed for resin regeneration, and able to retain capacity and selectivity over multiple cycles. Resin selection is determined by recombinant protein properties such as charge, hydrophobicity, and biospecificity. Selecting a resin based on the property most unique to the recombinant protein compared to the host system can improve purification efficiency by increasing binding capacity and/or product purity. Once suitable chromatography resin functionality is determined (cation/anion-exchange, affinity, hydrophobic), various resins with different particle sizes, surface areas, and resin backbones need to be screened along with binding conditions, such as pH and ionic strength. [1].

The manufacturing cost for plant-produced proteins consists of upstream (biomass production) and downstream purifications costs. The downstream processing costs are also affected by the ease of product recovery, the complexity

of clarified plant or cell culture extracts, protein stability, and required purity [94]. The support activities, such as process and cleaning validation, buffer preparation, equipment cleaning, and quality control and quality assurance, could be a substantial fraction of operating costs. From a downstream cost perspective, several evaluation criteria could be applied for selecting the best system that matches product characteristics and allows overall manufacturing cost reduction. In addition to protein expression level and stability, the following criteria should be considered: biomass yield and storage stability (allowing processing flexibility and decoupling), selection of off-the-shelf purification tools, biomass disposal cost, and byproduct revenues (e.g. biomass and starch conversion to energy) [1].

3.2 Upstream processing

The commercial and economic success of any plant-based production platform for recombinant proteins depends on the product yield, since the costs of upstream production relate to the amount of biomass produced, and the greater the amount of product per unit of biomass the better.

The subcellular localization of a protein contributes to its stability, so further yield improvements have been achieved by adding targeting sequences, such as a signal peptide to allow secretion (into the medium for plant cells, or to the apoplast in whole plants), a signal peptide and a KDEL/HDEL tetrapeptide so that secreted proteins are retrieved to the ER, or a signal peptide and a transmembrane domain so the recombinant protein is concentrated in the membrane fraction[95]. Another strategy involves the expression of recombinant proteins as fusions with stabilizing sequences, such as the elastin-like peptide repeat, which not only increases yields but also provides a convenient extraction method known as reverse transition cycling (reversible temperature-dependent precipitation) [12].

The initial step in the process usually involves milling of the tissue in either a dry state or together with an aqueous extraction buffer. In some cases, this may be preceded by a dehulling step or separation of endosperm and germ (corn) to remove a portion of the biomass that is largely devoid of the recombinant protein. If a dry milling process is employed, it is usually followed by a separate aqueous extraction step. The method of choice may be dependent on seed type, target protein and nature of the process. For an undisclosed protein expressed in corn, it was found that wet milling resulted in product losses and so the two-step dry milling and extraction process was preferable [96]. Extraction of the tissue is generally followed by some form of filtration or centrifugation to remove solids that can represent 5%–30% of the total mass of the slurry. At this stage, in most seedbased processes, it is often desirable to perform a concentration step to reduce volumes prior to advancing into chromatography. The safflower oilbody process differs here in that the extract consists of an emulsion in which the recombinant protein is bound to seed oilbodies. The oilbodies and bound protein are separated from the majority of endogenous seed protein through a series of flotation–centrifugation steps. The washed oilbody fraction is then treated to either elute or cleave off the recombinant protein and the oilbodies removed [28].

4. Legal regulations

The potential to use agronomically important food/feed commodity crops and other seed crops for non-food/feed GM-based applications such as biofuel enzymes or pharmaceuticals raises several regulatory issues for both regulatory agencies and consumers. Regulatory compliance for field growth (e.g., USDA APHIS/EPA) will encompass concepts of product segregation and stewardship. Crops expressing therapeutics or vaccines will likely remain under regulatory oversight for the life of the product and require containment protocols and documentation at each step in the process. In contrast, for industrial enzymes which are/will be produced at large scale, producers will likely seek deregulation. One of the regulatory considerations for food-based grains is that while there is the need to segregate the bioproduct source grain from the food/feed supply chain, they do have a G.R.A.S. status from the US Food and Drug Administration (FDA) which may reduce downstream hurdles linked with pharmaceutical use of these plant-based products [97].

Both US (FDA) and European (EMEA) regulatory authorities have recently published guidance documents on the production of therapeutic proteins from genetically engineered plants providing a framework for manufacturing in these systems [98,99]. The documents cover all aspects of the process from generation of the bioengineered plant through propagation and final purification. As the part of the process that differs most from that of conventional cell-based systems, considerable attention is given to production of the plant material used for manufacturing.

In 2005, the World Health Organization (WHO) conducted an ‘informal consultation on scientific basis for regulatory evaluation of candidate human vaccines from plants’[100]. In its report, the WHO recommended that the guidelines on Good Agricultural and Collection Practices (GACP), which are typically applied to herbal plants, should also be applied to plants producing biopharmaceuticals. A report of quality-control methods for medicinal plant materials recommended tests to assess the identity, purity, and content of biopharmaceutical plant materials. [101].

Since 2008, the United States Department of Agriculture (USDA) has approved field release of transgenic seeds expressing human lysozyme, lactoferrin, and serum albumin in rice, apolipoprotein in safflower, and hepatitis B surface antigen and brazzein in corn.

5. Conclusions

Since plant cell cultures are inherently simpler, cheaper and safer than mammalian cell approaches and support eukaryotic processing (e.g. complex glycosylation) absent in microbial systems, this technology has continued to attract attention as an alternative host system. For the process of recombinant proteins, most suitable subcellular compartment or organelle should be selected for easy recombinant protein extraction; protein product and plant extract properties should be match for optimal purification; for oral vaccines, plants should be selected with GRAS status, it should benefit chloroplast expression for high expression levels.

References

[1] Wilken L, Nikolov Z. Recovery and purification of plant-made recombinant proteins. Biotechnology Advances. 2012; 30: 419-33.

[2] Xu JF, Ge XM, Dolan MC. Towards high-yield production of pharmaceutical proteins with plant cell suspension cultures. Biotechnology Advances 2011; 29:278-99.

[3] De Muynck B, Navarre C, Boutry M. Production of antibodies in plants: status after twenty years. Plant Biotechnology Journal. 2010;8: 529-63.

[4] Gomord V, Fitchette AC, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, Michaud D, Faye L. Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnology Journal. 2010;8:564-87.

[5] Obembe OO, Popoola JO, Leelavathi S, Reddy SV. Advances in plant molecular farming. Biotechnology Advances. 2011;29:210-22.

[6] Paul M, Ma JKC. Plant-made pharmaceuticals: leading products and production platforms Biotechnology Applied Biochemistry. 2011;58:58-67.

[7] Pujol M, Ramirez NI, Ayala M, Gavilondo JV, Valdes R, Rodriguez M, Brito J,Padilla S,Gómez L,Reyes B,Peral R,Pérez M,Marcelo JL,Milá L,Sánchez RF,Páez R,Cremata JA,Enríquez G,Mendoza O,Ortega M,Borroto C. An integral approach towards a practical application for a plant-made monoclonal antibody in vaccine purification. Vaccine 2005;23:1833-7.

[8] Hood EE. From green plants to industrial enzymes. Enzyme Microbiology Technology .2002;30: 279-83.

[9] Scheller J, Guhrs KH, Grosse F, Conrad U. Production of spider silk proteins in tobacco and potato. Nature Biotechnology 2001;19:573-7.

[10] Eisoldt L, Smith A, Scheibel T. Decoding the secrets of spider silk. Mater Today 2011;14: 80-6.

[11] Conley AJ, Joensuu JJ, Jevnikar AM, Menassa R, Brandle JE. Optimization of elastin-like polypeptide fusions for expression and purification of recombinant proteins in plants. Biotechnology Bioengineering 2009;103:562-73.

[12] Conley AJ, Joensuu JJ, Richman A, Menassa R. Protein body-inducing fusions for highlevel production and purification of recombinant proteins in plants. Plant Biotechnology Journal 2011;9:419-33

[13] Xu J, Dolan MC, Medrano G, Cramer CL, Weathers PJ. Green factory: Plants as bioproduction platforms for recombinant proteins. Biotechnology Advances 2012;30 :1171-84

[14] Ma S, Wang A. Molecular farming in plants: An overview. Molecular farming in plants: Recent advances and future prospects. 2012; 1-20.

[15] Odell J, Nagy,F, Chua N. Identification of dna-sequences required for activity of the cauliflower mosaic virus-35s promoter. Nature. 1985; 313: 810-12.

[16] Streatfield S. Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnology Journal.2007; 5: 2-15.

[17] Available online https://hpcwebapps.cit.nih.gov/dnaworks.

[18] Available online -http://genomes.urv.cat/OPTIMIZER/

[19] Hood E, Vicuna Requesens D. Recombinant protein production in plants: challenges and solutions. Methods in Molecular Biology: Recombinant Gene Expression Humana Press, New York. 2011.

[20] Ma S, Huang Y, Davis A, Yin Z, Mi Q, Menassa R, Brandle J, Jevnikar A. Production of biologically active human interleukin-4 in transgenic tobacco and potato. Plant Biotechnology Journal. 2005; 3: 309-18.

[21] Hondred D, Walker J, Mathews D, Vierstra R. Use of ubiquitin fusions to augment protein expression in transgenic plants. Plant Physiology. 1999; 119: 713-23.

[22] Coleman CE, Larkins BA. 6. The Prolamins of Maize. seed Proteins. 1999. p.109.

[23] Liu Q, Singh S, Green A. High-oleic and high-stearic cottonseed oils: Nutritionally improved cooking oils developed using gene silencing. Journal of the American College of Nutrition. 2002; 21: 205-11.

[24] Kinney A, Jung R, Herman E. Cosuppression of the alpha subunits of betaconglycinin in transgenic soybean seeds induces the formation of endoplasmic reticulum-derived protein bodies. Plant Cell. 2001; 13: 1165-78.

[25] Kermode A. Plants as factories for production of biopharmaceutical and bioindustrial proteins: lessons from cell biology. Canadian Journal of Botany- Revue Canadienne De Botanique. 2006; 84: 679-94.

[26] Wu Y, Messing J. RNA Interference can rebalance the nitrogen sink of maize seeds without losing hard endosperm. Plos One. 2012; 7.

[27] Houmard NM, Mainville JL, Bonin CP. Huang S, Luethy MH, Malvar TM. High lysine corn generated by endosperm specific suppression of lysine catabolism using RNAi. Plant Biotechnology Journal. 2007; 5: 605-14.

[28] Boothe J, Nykiforuk C, Shen Y, Zaplachinski S, Szarka S, Kuhlman P, Murray E, Morck D, Moloney MM. Seed-based expression systems for plant molecular farming Plant Biotechnology Journal 2010; 8: 588-606.

[29] Fischer R, Schillberg S, Twyman RM Molecular farming of antibodies in plants. Recent Advances in Plant Biotechnology New York: Springer. 2009; 35-63.

[30] Schillberg S, Twyman R, Fischer R. Opportunities for recombinant antigen and antibody expression in transgenic plants - technology assessment. Vaccine.2005; 23: 1764-69.
[31] Huang N. High-level protein expression system uses selfpollinatingcrops as hosts. BioProcess International. 2004; 2: 54-59.
[32] Haq TA, Mason HS, Clements JD, Arntzen CJ. Oral immunization with a recombinant bacterial antigenproduced in transgenic plants. Science.1995; 268: 714-16.
[33] Mason HS, Ball JM, Shi JJ, Jiang X, Estes MK, Arntzen CJ.Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicityin mice.Proceedings. National Academy Science of the USA.1996; 93:5335-40.
[34] Tacket CO, Mason HS, Losonsky G, Estes MK, Levine MM, Arntzen CJ. Human immune responses to a novel norwalk virus vaccine delivered in transgenic potatoes. Journal of Infectious Diseases. 2000;182:302-05.
[35] Castanon S, Marin MS, Martin-Alonso JM, Boga JA, Casais R, Humara JM, Ordas RJ, Parra F. Immunization with potato plants expressing VP60 proteinprotects against rabbit hemorrhagic disease virus. Journal of Virology.1999;73:4452-55.
[36] Richter LJ, Thanavala Y, Arntzen,CJ, Mason HS. Production of hepatitis B surface antigen in transgenic plantsfor oral immunization. Nature Biotechnology. 2000; 18: 1167-71.
[37] Kong Q, Richter L, Yang YF, Arntzen CJ, Mason HS, Thanavala Y. Oral immunization with hepatitis B surface antigen expressed in transgenic plants. Proceedings of National. Academy of Science. 2001;98: 11539-44.
[38] Yu J, Langridge WH. A plant-based multicomponent vaccine protects mice from enteric diseases. Nature Biotechnology. 2001;19:548-52.
[39] Franconi R., Di BP , Dibello F, Accardi L, Muller A, Cirilli A, Simeone P, Dona MG, Venuti A, Giorgi C. Plant-derived human papillomavirus 16 E7 on coprotein induces immune response and specific tumor protection. Cancer Research. 2002; 62: 3654-58.
[40] Biemelt S, Sonnewald U, Galmbacher P, Willmitzer L, Muller M. Production of human papillomavirus type 16virus-like particles in transgenic plants. Journal of Virology. 2003;77:9211-20.
[41] Warzecha H, Mason HS, Lane C, Tryggvesson A, Rybicki E,Williamson AL, Clements JD, Rose RC. Oral immunogenicity of human papillomavirus-like particles expressed in potato Journal of Virology. 2003; 77:8702-11.
[42] Berinstein A, Vazquez-Rovere C, Asurmendi S, Gomez E, Zanetti F, Zabal O, Tozzini A, Conte GD, Taboga O, Calamante G, Barrios H, Hopp E, Carrillo E. Mucosal and systemic immunization elicited by Newcastle disease virus (NDV) transgenic plants as antigens. Vaccine 2005; 23: 5583-89.
[43] Arntzen C. Plant-made pharmaceuticals:From“EdibleVaccines” to Ebola therapeutics. Plant Biotechnology. 2015; 13:1013-16.
[44] Planet Biotechnology Inc. Home Page. Availableonline: http://www.planetbiotechnology.com
[45] Mason HS, Lam DM, Arntzen CJ. Expression of hepatitis B surface antigen in transgenic plants. Proceedings of National. Academy of Science. 1992;89: 11745-49.
[46] Barta A, Sommengruber K, Thompson D, Hartmuth K, Matzke M, Matzke A. The expression of a napoline synthase human growth hormone chimeric gene in transformed tobacco and sunflower callus tissue. Plant Molecular Biology. 1986;6: 347-57.
[47] Fox JL. First plant-made biologic approved. Nature Biotechnology. 2012; 30.
[48] Shaaltiel Y, Bartfeld D, Hashmueli S, Baum G, Brill-Almon E, Galili G, Dym O, Boldin-Adamsky SA, Silman I, Sussman, JL, Futerman AH, Aviezer D. Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. Plant Biotechnology of Journal. 2007; 5: 579-90.
[49] Aviezer D , Brill-Almon E, Shaaltiel Y, Hashmueli S, Bartfeld D, Mizrachi S, Liberman Y, Freeman A, Zimran A, Galun E. A plant-derived recombinant human glucocerebrosidase enzyme--a preclinical and phase I investigation. PLoS One online-only. 2009; 4;3:4792.
[50] Antibodies-online Inc.Home Page.Available online: http://www.antibodies-online.com
[51] Promo Kine Home Page. Available online: https://www.promokine.info
[52] Yao J, Weng Y, Dickey A, Wang KY. Plants as factories for human pharmaceuticals: Applications and challenges. International Journal of Molecular Sciences. 2015; 16: 28549-65.
[53] Zeitlin L, Olmsted SS, Moench TC, Co MS, Martinell BJ, Paradkar VM, Russell DR, Queen C, Cone RA, Whaley K.A. Humanized monoclonal antibody produced in transgenic plants for immuno protection of the vagina against genital herpes. Nature Biotechnology. 1998; 16: 1361-64.
[54] Tan Y, Wang KY, Wang N, Li G, Liu D. Ectopic expression of human acidic fibroblast growth factor 1 in the medicinal plant, *Salvia miltiorrhiza*, accelerates the healing of burn wounds. BMC Biotechnology. 2014; 14:74
[55] Available online http://www.sembiosys.com
[56] Cox K, Sterling M, Regan JD, Gasdaska J T, Frantz J R , Peele KK, Black CG, Passmore A, Moldovan-Loomis D, , Srinivasan C, Cuison M, Cardarelli S, P. M., Dickey L.F. Glycan optimization of a human monoclonal antibody in the aquatic plant Lemna minor. Nature Biotechnology. 2006;24: 1591-97.
[57] Available online http://www.plantpharma.org/ 2005/06/state-shouldn't-miss -opportunities-with-agragen
[58] Available online http://www.ventria.com
[59] Nandi S, Suzuki YA, Huang J, Yalda D, Pham P, Wu L, Bartley G, Huang N, Lonnerdal B. Expression of human lactoferrin in transgenic rice grains for the application in infant formula. PlantScience2002; 163:713-22.
[60] http://web.archive.org/web/ 20071012232910/www.meristem -therapeutics.com/rubrique.php3?idrubrique=64
[61] http://web.archive.org/web/20071012232733/www.meristemtherapeutics.com/rubrique.php3? idrubrique=38
[62] Bor MV, Fedosov SN, Laursen NB, Nexo E. Recombinant human intrinsic factor expressed in plants is suitable for use in measurement of vitamin B12. Clinical Chemistry. 2003;49: 2081-83.
[63] Fedosov SN, Laursen NB, Nexo E, Moestrup SK, Petersen TE, Jensen EO, Berglund L. Human instrinsic factor expressed in the plant *Arabidopsis thaliana*. European Journal of Biochemistry. 2003;270: 3362-67.
[64] Available online http://www.medicago.com Clinical trial NCT00984945[

[65] Thanavala Y, Mahoney M, Pal S, Scott A, Richter L, Natarajan N, Goodwin P, Arntzen C J, Mason H.S. Immunogenicity in humans of an edible vaccine for hepatitis B. Proceedings. National Academy Science of the USA. 2005;102: 3378-82.
[66] Kapusta J, Modelska A, Figlerowicz M, Pniewski T, Letellier M,Lisowa O, Yusibov V, Koprowski H, Plucienniczak A, Legocki AB. A plant-derived edible vaccine against hepatitis B virus. FASEB Journal. 1999; 13: 1796-99.
[67] Yusibov V, Hooper DC, Spitsin SV, FleyshN, Kean RB, Mikheeva T, Deka D, Karasev A, Cox S, Randall J, Koprowski H. Expression in plants and immunogenicity of plant virus-based experimental rabies vaccine. Vaccine 2002; 20: 3155-64.
[68] Available online http://www.bayer-innovation.com
[69] Bendandi M, Marillonnet S, Kandzia R, Thieme F, Nickstadt A, Herz S, Frode R, Inoges S, Lopez-Diaz de Cerio A, Soria E, Villanueva H, Vancanneyt G, McCormick A, Tuse D, Lenz J, Butler-Ransohoff JE, Klimyuk V, Gleba Y. Rapid, high-yield production in plants of individualized idiotype vaccines for non-Hodgkin's lymphoma. Annals of Oncology. 2010; 21: 2420-27
[70] Morandini F, Avesani L, Bortesi L. Non-food/feedseeds as biofactories for the high-yield production of recombinant pharmaceuticals. Plant Biotechnology Journal .2011; 9: 911–21.
[71] Loos A,Van Droogenbroeck B, Hillmer S. Grass J, Kunert R, Cao J, Robinson DG, Depicker A, Steinkellner H. Production of monoclonal antibodies with a controlled *N*-glycosylation pattern in seeds of Arabidopsis thaliana. Plant Biotechnology Journal. 2011; 9: 179192.
[72] Sch¨ahs M, Strasser R, Stadlmann J, Kunert R, Rademacher T, Steinkellner H. Production of a monoclonal antibody in plants with a humanized *N*-glycosylation pattern. Plant Biotechnology Journal.2007;5: 657-63.
[73] Ramessar K, Rademacher T, Sack M. Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. Proceedings of the National Academy of Sciences of the United States of America .2008; 105:3727–32
[74] Lai H, He J, Engle M, Diamond MS, Chen Q. Robust production of virus-like particles and monoclonal antibodies with Gemini viral replicon vectors in lettuce. Plant Biotechnology Journal.2012; 10: 95-104.
[75] Zhang X, Buehner NA, Hutson AM, Estes MK, Mason HS. Tomato is a highly effective vehicle for expression and oral immunization with Norwalk virus capsid protein. Plant Biotechnology Journal .2006; 419-32
[76] Porcedd A, Falorni A, Ferradini N. Transgenic plants expressing human glutamic acid decarboxylase (GAD65), a major auto antigen in insulin-dependent diabetes mellitus. Molecular Breeding. 1999; 5: 553-60.
[77] Menassa R,Nguyen V, Jevnikar AN, Brandle JE. A self-contained systemfor the field production of plant recombinant human interleukin-10. Molecular Breeding 2001;8:177-85.
[78] Fischer R, Stoger E, Schillberg S, Christou P, Twyman RM. Plant-based production of biopharmaceuticals. Current Opinion in Plant Biology 2004;7:152-8.
[79] Specht E, Miyake-Stoner S, Mayfield S. Micro-algae come of age as a platform for recombinant protein production.Biotechnology Letters.2010; 10: 1373-83.
[80] Robert S, Goulet MC, D'Aoust MA, Sainsbury F, Michaud D. Leaf proteome rebalancing in Nicotiana benthamiana for upstream enrichment of a transiently expressed recombinant protein Plant Biotechnology Journal. 2015;13 1169-79.
[81] Circelli P, Donini M, Villani ME, Benvenuto E, Marusic C. Efficient Agrobacterium-based transient expression system for the production of biopharmaceuticals in plants. Bioengineered Bugs 2010;1:221-4.
[82] Matoba N, Davis KR, Palmer KE. Recombinant protein expression in Nicotiana. Methods in Molecular Biology 2011;701:199-219.
[83] Medrano G, Dolan MC, Stephens NT, McMickle A, Erf G, Radin D, Cramer CL. Efficient plantbased production of chicken interleukin-12 yields a strong immunostimulatory cytokine. Journal of Interferon & Cytokine Research 2010;30:143-54.
[84] Marillonnet S, Giritch A, Gils M, Kandzia R, Klimyuk V, Gleba Y. In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by Agrobacterium. Proceedings of the National Academy of Science U S A 2004;101:6852-7.
[85] Shanks JV, Morgan J. Plant 'hairy root' culture. Current Opinion in Biotechnology 1999;10:151-5.
[86] Harrison RG, Todd P, Rudge SR, Petrides DP. Bioprocess design. In: Gubbins KE, editor. Bioseparations science and engineering. New York, NY: Oxford University Press, Inc.; 2003. p. 319-72.
[87] Moeller L, Taylor-Vokes R, Fox S, Gan Q, Johnson L, Wang K. Wet-milling transgenic maize seed for fraction enrichment of recombinant subunit vaccine. Biotechnology Progress 2010;26:458-65.
[88] Nikolov ZL, Regan JR, Dickey LF, Woodard SL. Purification of antibodies from transgenic plants. In: Gottschalk U, editor. editor. Process scale purification of antibodies: Wiley-VCH; 2009. p. 387-406.
[89] Nykiforuk CL, Shen Y, Murray EW, Boothe JG, Busseuil D, Rhéaume E, et al. Expression and recovery of biologically active recombinant Apolipoprotein AIMilano from transgenic safflower (Carthamus tinctorius) seeds. Plant Biotechnology 2011;9:250-63.
[90] Azzoni AR, Farinas CS, Miranda EA. Transgenic corn seed for recombinant protein production: relevant aspects on the aqueous extraction of native components. Journal Science Food Agriculture. 2005;85:609-14.
[91] Balasubramaniam D, Wilkinson C, Van Cott K, Zhang C. Tobacco protein separation by aqueous two-phase extraction. Journal of Chromatography A 2003;989:119-29.
[92] Azzoni AR, Kusnadi AR, Miranda EA, Nikolov ZL. Recombinant aprotinin produced transgenic corn seed: extraction and purification studies. Biotechnology Bioengineering 2002;80:268-76.
[93] Wilken LR, Nikolov ZL. Evaluation of alternatives for human lysozyme purification from transgenic rice: impact of phytic acid and buffer. Biotechnology Progress 2010;26: 1303-11.
[94] Nikolov ZL, Hammes D. Production of recombinant proteins from transgenic crops. In: Hood EE, Howard JA, editors. Plant as factories for protein production. Dordrecht, The Netherlands: Kluwer Academic Publishers; 2002. p. 159-74.
[95] Sharma AK, Sharma MK. Plants as bioreactors: recent developments and emerging opportunities. Biotechnology Advances 2009;27:811-32.
[96] Menkhaus TJ, Bai y, Zhang C-M, Nikolov ZL, Glatz CE. Considerations for the recovery of recombinant proteins from plants. Biotechnology Progress. 2004: 1001-14.

[97] Fischer R, Schillberg S, Hellwig S, Twyman RM, Drossard J. GMP issues for recombinant plant-derived pharmaceutical proteins Biotechnology Advances. 2012;30: 434-39
[98] FDA. Guidance for industry: drugs, biologics, and medical devices derived from bioengineered plants for use in humans and animals. Draft Guidance. 2002.
[99] EMEA (2008) Guideline on the quality of biological active substances produced by stable transgene expression in higher plants. Doc. Ref. EMEA/CHMP/BWP/48316/2006.
[100] van der Laan J, Minor P, Mahoney R, Arntzen C, Shin J, Wood D, WHO Informal Consultation Group. WHO informal consultation on scientific basis for regulatory evaluation of candidate human vaccines from plants, Geneva, Switzerland, 24–25 January 2005. 2006. Vaccine 24: 4271-78.
[101] Takeyama N, Kiyono H, Yuki Y. Plant-based vaccines for animals and humans: recent advances in technology and clinical trials. 2015. 3;5-6: 139-54.

Vitamin B6: a heart-protective molecule through a novel mechanism of increasing histidine-related compounds

Thanutchaporn Kumrungsee[1], **Dwi Eva Nirmagustina**[1], **Norihisa Kato**[1], **Noriyuki Yanaka**[1] and **Hong C. Lai**[2]

[1] Graduate School of Biosphere Science, Hiroshima University, 7398528 Higashi-Hiroshima, Japan
[2] School of Medicine, Johns Hopkins University, Baltimore, MD, United States

It has been reported that a low dietary intake of vitamin B6 is associated with a high risk of mortality from heart disease among Japanese, Americans, and Europeans [1]. Many studies have suggested that vitamin B6 supplementation reduces a risk of heart disease [1–3]. However, the relationship of vitamin B6 intake and risk of heart disease is controversial, and the underlying mechanisms are not fully understood. Since 1969, the role of vitamin B6 in heart disease risk has been mostly addressed through homocysteine hypothesis [4]. However, many recent studies have indicated that the association between vitamin B6 and heart disease risk has been independent of homocysteine, suggesting that vitamin B6 may exert a heart-protective effect through other mechanisms [2,4]. Recently, we found that vitamin B6 supplementation in rats had no effect on homocysteine concentrations in the heart tissue, but markedly increased heart concentrations of histidine-related compounds, such as carnosine, anserine, and histamine [5]. Thus, in the present chapter we propose a novel mechanism underlying heart-protective effect of vitamin B6 and review the currently available knowledge.

Keywords: vitamin B6; histidine-related compounds; carnosine; histamine; heart; cardiovascular disease

1. Introduction

Vitamin B6 is an essential water-soluble vitamin. In humans, our body is lack of an ability to produce this vitamin as well as to store it to long extent due to its water-soluble property. Thus, in order to keep balance of vitamin B6 status, we mainly rely on food consumption. Vitamin B6 can be found in various foods such as fish, beef, poultry, milk products, whole grains, legumes, potatoes, and nuts. There are six isoforms of vitamin B6, which are pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM), and their phosphorylated forms of pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP), and pyridoxamine 5'-phosphate (PMP) (Fig. 1). Among those six isoforms, PLP is the most active form that acts as a co-factor or coenzyme in more than 150 enzymatic reactions including amino acid, fatty acid, and carbohydrate metabolisms. For example, PLP is important for enzymes responsible for syntheses of niacin, alanine, histamine, and γ-aminobutyric acid (GABA) from tryptophan, pyruvate, histidine, and glutamic acid, respectively. In fatty acid metabolism, PLP is a co-factor of enzymes responsible for polyunsaturated fatty acid synthesis. In carbohydrate metabolism, PLP is important for generating energy of our body by helping enzymes to breakdown storage carbohydrates to glucose. Being involved in diverse enzymatic reactions, vitamin B6 deficiency has been reported to be associated with an increased risk of many diseases including cardiovascular disease or heart disease [2].

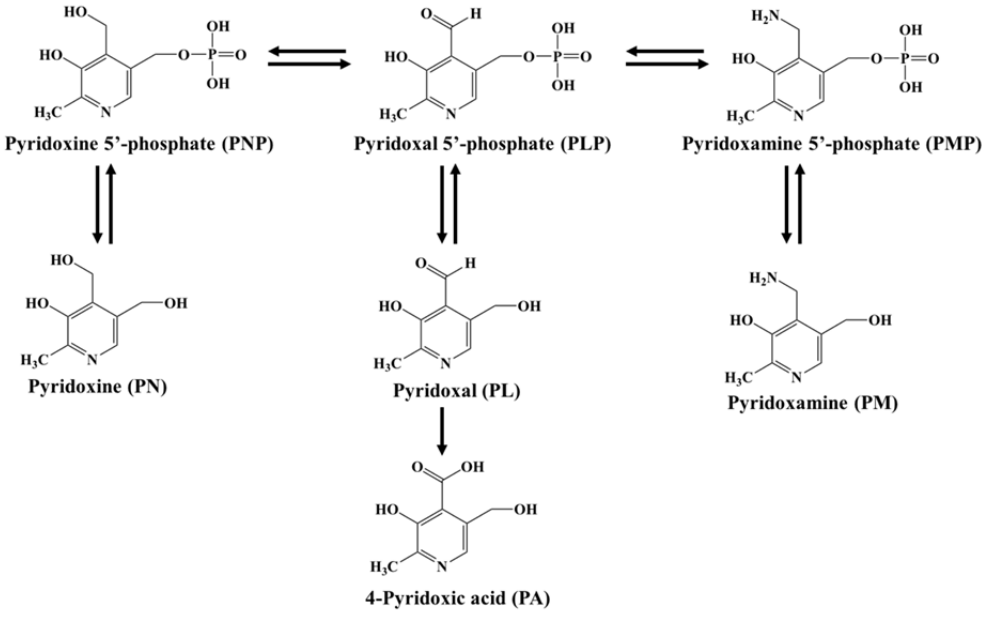


Fig. 1 Vitamin B6 forms. In food, vitamin B6 is existing in PN form. After being absorbed into our body, PN is changed to PLP (the most active form) and/ or PMP, which act as co-factors or co-enzymes.

2. Vitamin B6 and heart disease

After discovery of vitamin B6 in 1934, 15 years later the association of vitamin B6 with heart disease was reported. In 1949, Rinehart and Greenberg [6] demonstrated that monkeys fed a diet with vitamin B6 deficiency had atherosclerosis, a major condition leading to heart disease. Later on, many studies were conducted extensively to address this issue. In 1985, plasma PLP level was proposed to be a risk index for coronary artery disease (CAD), in which low level of PLP in plasma was found to be associated with a high risk of CAD [7]. Both animal and human studies have suggested that vitamin B6 supplementation reduces a risk of various types of heart disease [2,3]. The role of the vitamin in heart disease risk has been mostly addressed through homocysteine and inflammation hypotheses as reviewed below.

2.1 Homocysteine hypothesis

Homocysteine is a sulphur-containing amino acid, which is derived from metabolism of methionine. In general, homocysteine plasma levels increase after meals, especially meals rich in red meat and milk products. Our body maintains homocysteine homeostasis through two main pathways, which are remethylation, in which homocysteine receives a methyl group and is reconverted into methionine, and transulphuration, in which it is degradated to cysteine [4], as shown in Fig. 2. In transulphuration pathway, vitamin B6 plays a crucial role as a coenzyme of cystathionine β-synthetase for the conversion of homocysteine to cystathionine and of cystathionase for the synthesis of cysteine from cystathionine [4]. Thus, vitamin B6 deficiency leads to the accumulation of homocysteine in the body.

Since 1969, homocysteine has been reported to be involved in the pathophysiology of the atherosclerotic process [8]. Since then, many studies have suggested the correlation of elevated homocysteine with cardiovascular risk and established homocysteine as an independent risk factor. Increased homocysteine plasma levels promote blood clots and initiation of endothelial cell damage, which leads to inflammation in the blood vessels [8]. Then, inflammation induces atherogenesis that is an important factor causing blockage of blood flow, which finally contributes to heart disease such as heart attack. Based on the fact that vitamin B6 is a key determinant of total plasma homocysteine concentrations, dietary supplement of vitamin B6 has been proposed for many years to use as a tool for reducing plasma homocysteine levels and incidence of heart disease.

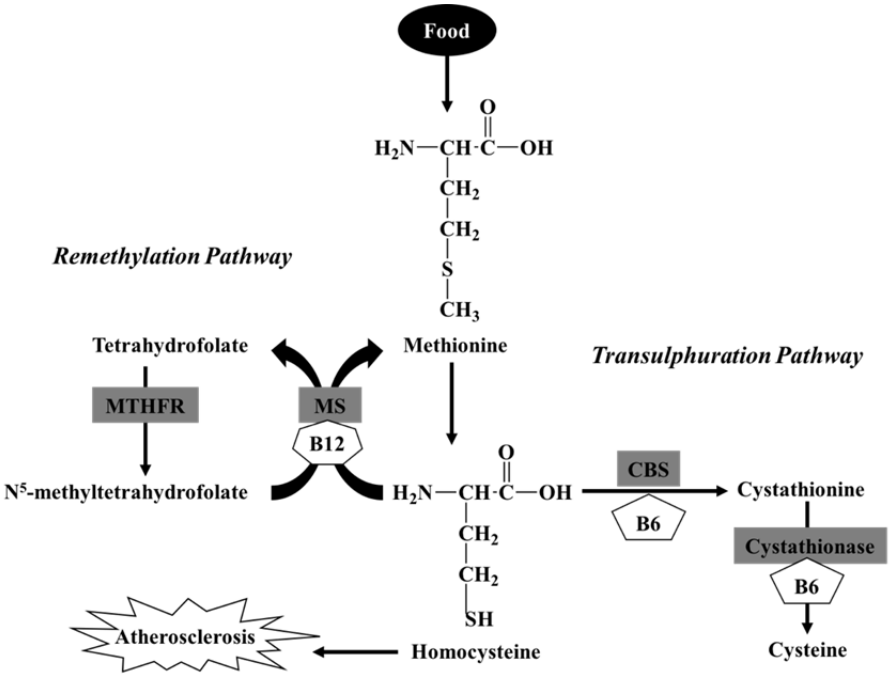


Fig. 2 Homocysteine metabolism. MTHFR: methylene tetrahydrofolate reductase; MS: Methionine Synthase; CBS: Cystathione β-synthase.

2.2 Inflammation hypothesis

Over recent 20 years, vitamin B6 has been proposed to play a role in heart disease through inflammation, which is believed to be a crucial mechanism underlying atherosclerosis and heart disease progression. Many epidemiological studies have demonstrated a strong relationship between vitamin B6 status and inflammation, in which plasma PLP concentrations were inversely correlated with inflammation markers such as C-reactive protein (CRP) [9–13]. A possible mechanism is that vitamin B6 or PLP serves as co-factor in an anti-inflammatory mechanism. When inflammation occurs, vitamin B6 from liver and peripheral tissues is mobilized to the inflammatory sites and acts as a coenzyme in pathways producing metabolites exerting anti-inflammatory or immunomodulatory effects. The use of

vitamin B6 in inflammatory processes results in a decrease in the vitamin concentrations in the body, which could promote and enlarge inflammatory process, thereby leading to chronic inflammatory disease progression. Based on scientific evidences, including human studies, it is unclear whether inflammation induces vitamin B6 deficiency in the body or vitamin B6 deficiency, due to inadequate vitamin B6 intake, induces inflammation. However, it could be said that vitamin B6 deficiency could possibly contribute to impairment of an anti-inflammatory mechanism, consequently leading to chronic inflammation and establishment of heart disease.

Recent research has been focused on clarifying underlying mechanisms in vitamin B6-dependent inflammatory pathways. One of the main pathways is the kynurenine pathway, which involved in tryptophan metabolism [13] as shown in Fig. 3. In this pathway, PLP acts as a co-factor of enzymes that convert kynurenine into a variety of compounds, including kynurenic acid, antranilic acid, xanthurenic acid, and 3-hydroxyanthranilic acid. These kynurenine-related compounds have been reported to have various beneficial effects on anti-inflammatory mechanism.

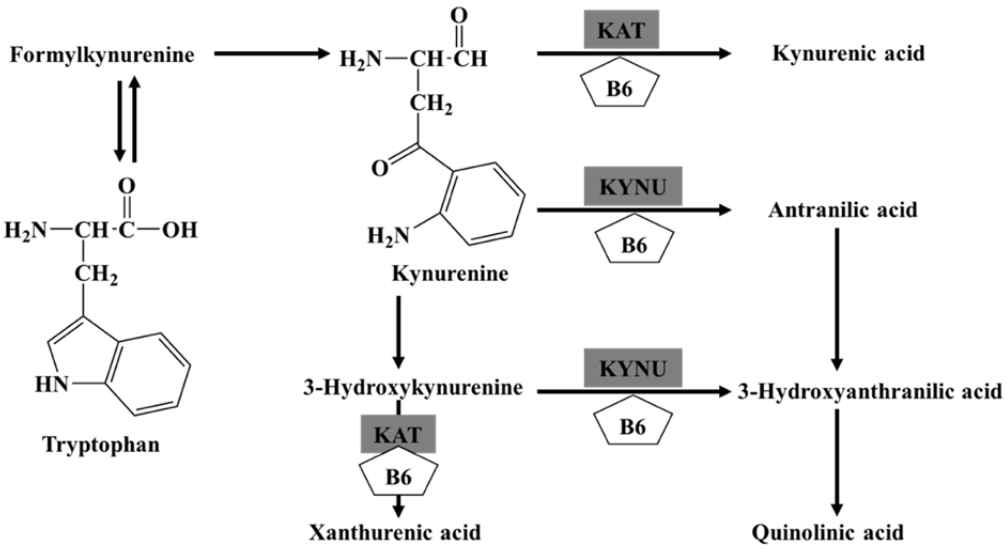


Fig. 3 Kynurenine pathway of tryptophan metabolism and PLP-dependent enzymes involved. KAT: kynurenine aminotransferase; KYNU: kynureninase.

2.3 Controversial in clinical studies

In spite of extensive studies since 1949, the link between vitamin B6 and heart disease is controversial, and the underlying mechanisms are not fully understood. Several large clinical trials have been designed to test the theory of vitamin B6 preventing heart disease, such as the Second Cambridge AntiOxidant Heart Study (CHAOS-2), the Vitamin Intervention for Stroke Prevention (VISP) trial, the Norwegian Vitamin (NORVIT) trial, the Heart Outcome Prevention Evaluation-2 (HOPE-2) trial, the Homocysteinemia in Kidney and End-Stage Renal Disease (HOST) trial, the Women's Antioxidant and Folic Acid Cardiovascular Study (WAFACS), the Western Norway B-vitamin Intervention Trial (WENBIT), and the Atherosclerosis Risk in Communities (ARIC) study [4]. These studies demonstrated inconsistent results. Many studies have reported that vitamin B6 had no preventive effects on heart disease and there was no inverse association between PLP levels and homocysteine and CRP levels. The controversial results may suggest a potential protective effect of vitamin B6 through mechanisms other than those related to homocysteine metabolism and inflammation.

3. Possible novel mechanism of heart-protective effects of vitamin B6 through histidine-related compounds

Since there are controversial data about heart-protective effects of vitamin B6, in our present study, we directly assessed metabolites in heart tissues of rats fed a normal diet supplemented with low, mild, or high concentration of vitamin B6 (1, 7, or 35 mg pyridoxine (PN) HCl/kg diet, respectively) for 6 weeks. As a result, all vitamin B6 supplementations had no effect on food intake, final body weight, and heart weight (data not shown). PLP concentrations in heart tissues and serum of the 7 and 35 mg PN HCl/kg groups were higher ($P < 0.01$) than those in the 1 mg PN HCl/kg group, while there was no significant difference in PLP concentrations between the 7 and 35 mg PN HCl/kg groups (data not shown). The preliminary study [5] demonstrated that the 7 and 35 mg PN HCl/kg groups had markedly higher concentrations of carnosine (+114 and +162%, respectively, $P < 0.01$, Fig. 4) and anserine (+89 and +101%, respectively, $P < 0.01$, Fig. 4) than those in the 1 mg PN HCl/kg group, while significant differences of those in the 7 and 35 mg PN HCl/kg groups were not observed. These preliminary results led us to apply metabolomics analysis for assessing metabolites in heart

tissues of rats fed a normal diet supplemented with the supplemental level (the 35 mg PN HCl/kg diet) and the marginal deficient level (the 1 mg PN HCl/kg diet). As a result, both vitamin B6 supplementations in rats had no effect on concentrations of homocysteine and kynurenine-related compounds, suggesting that vitamin B6 was not likely to play the role through these two pathways. Over 500 detected compounds, 13 compounds were found to be significantly different between the two groups (Table 1). Serine, alanine, leucine, isoleucine, valine, β-alanine, GABA, histamine, carnosine, 1-methyl histidine, anserine, and homocarnosine levels in the 35 mg PN HCl/kg group were significantly higher than those in the 1 mg PN HCl/kg group. Ornithine was the only compound that its level was lower in the 35 mg PN HCl/kg group than in the 1 mg PN HCl/kg group. Based on these results, we have proposed how vitamin B6 played a role in regulating the levels of those compounds as below.

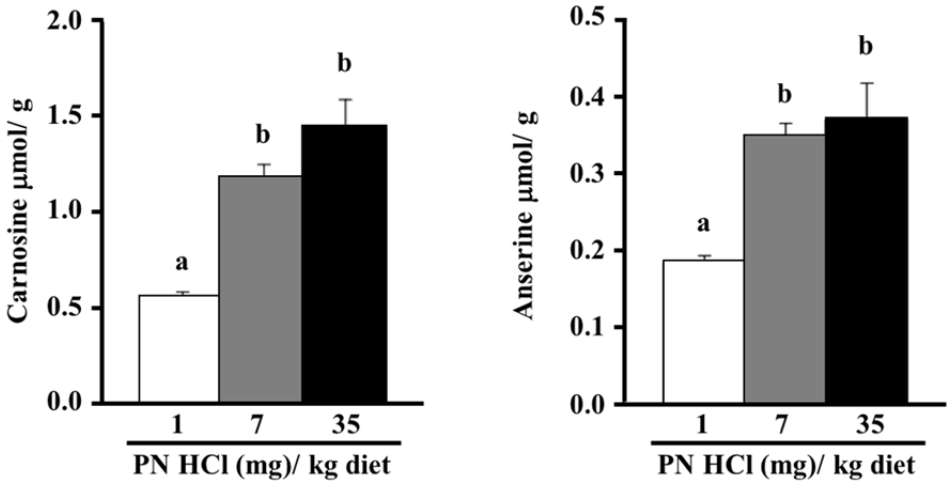


Fig. 4 Effect of dietary levels of vitaminB6 (PN HCl) on concentrations of carnosine and anserine in heart of rats. Mean ± SE (n = 4 for carnosine and anserine). The supernatant samples of each two rats from the same group of eight rats were combined to obtain the pooled four samples. Values with different superscript are significantly different by Tukey's multiple-range test ($P < 0.05$).

Table 1 Compounds with significant different levels detected in heart of rats fed low and high concentrations of vitamin B6 (PN HCl/ kg diet).

Compounds	Comparative Analysis (35 mg PN HCl/kg)/(1mg PN HCl/kg)	
	Ratio	P-value
Serine	1.43	< 0.05
Alanine	1.31	< 0.005
Leucine	1.12	< 0.05
Isoleucine	1.18	< 0.005
Valine	1.14	< 0.05
β-Alanine	1.89	< 0.001
GABA	2.28	< 0.005
Histamine	2.24	< 0.05
Carnosine	2.51	< 0.005
1-Methylhistidine	1.77	< 0.001
Anserine	2.07	< 0.001
Homocarnosine	1.90	< 0.005
Ornithine	0.76	< 0.005

As shown in Fig. 5, it seems likely that vitamin B6 induced the synthesis of histidine-related compounds (HRCs), which were histamine, carnosine, anserine, and homocarnosine. Among these four compounds, histamine is the only compound that its synthesis is directly depended on vitamin B6, since histidine decarboxylase is a PLP-dependent enzyme. For carnosine, anserine, and homocarnosine, these three compounds are the products of carnosine synthase.

Although carnosine synthase is not a PLP-dependent enzyme, it is possible that the vitamin may enhance the enzyme activity by increasing the levels of enzyme substrates, which are β-alanine and GABA. Since β-alanine is rate-limiting for carnosine synthesis [14], it can be speculated that an availability of GABA may be a factor limiting the rate of homocarnosine-synthesis as well. This speculation can be supported by a previous report suggesting that the availability of GABA greatly affected homocarnosine synthesis [15]. It seems reasonable to assume that vitamin B6 upregulates metabolism of amino acids that are reservoirs of β-alanine and GABA (Fig. 5). Vitamin B6 may regulate the levels of serine, alanine, leucine, isoleucine, and valine, which, in turn, play a role in maintaining homeostases of glutamic acid as well as aspartic acid. Then, vitamin B6 is directly involved in the formations of β-alanine and GABA by serving as a coenzyme of aspartate decarboxylase and glutamate decarboxylase, which were reported to be the rate-limiting steps in β-alanine and GABA formations [16,17]. In addition, vitamin B6 might be responsible for the degradation of ornithine to β-alanine and GABA by acting through ornithine decarboxylase and ornithine aminotransferase in polyamines and glutamic acid metabolic pathways. Taken together, we have hypothesized that vitamin B6 may positively influent enzymes involved in the formations of β-alanine and GABA, which are the rate-limiting precursors of histidine-related compounds.

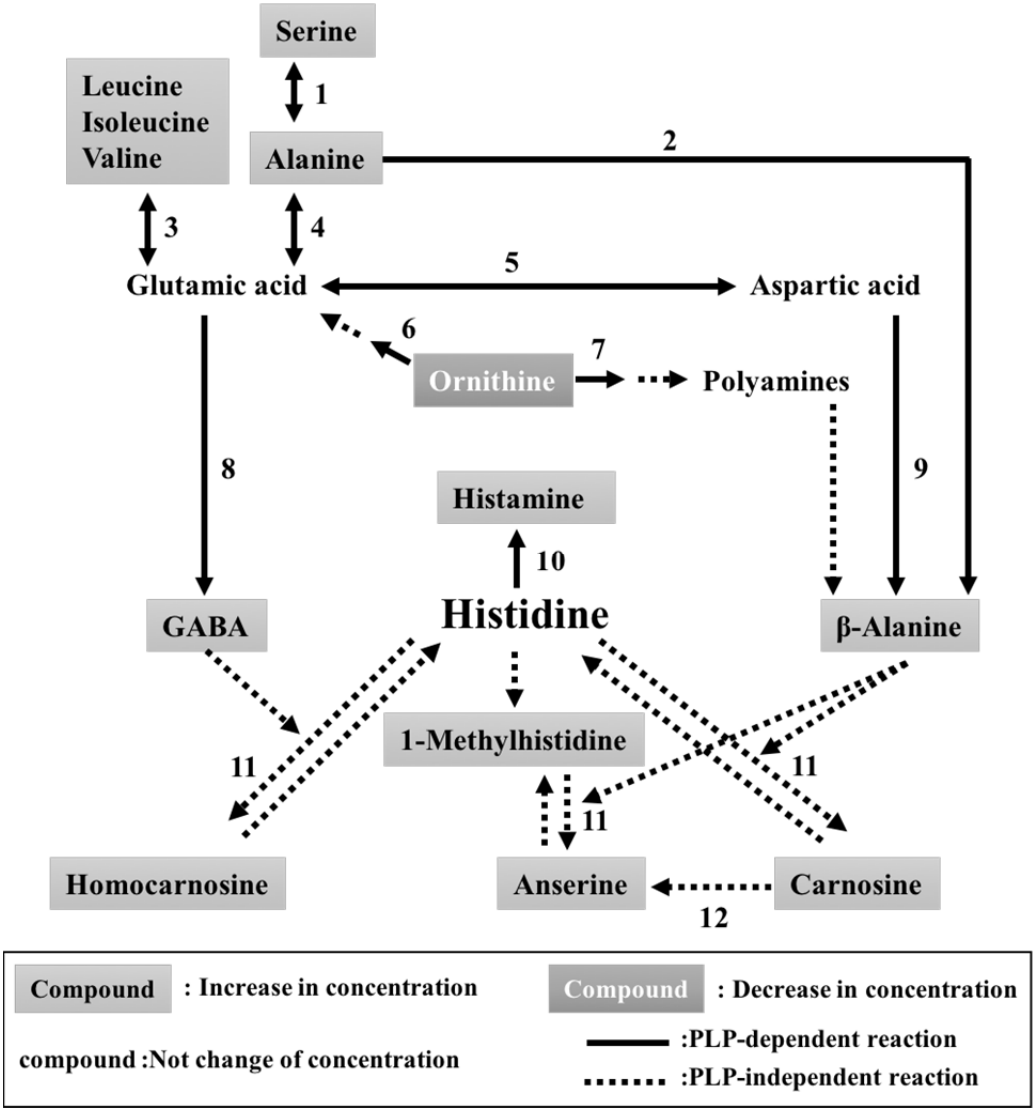


Fig. 5 Putative pathways of compounds affected by vitamin B6 in heat tissues of rats. 1: Serine-pyruvate aminotransferase; 2: β-alanine-pyruvate aminotransferase; 3: Branched-chain-amino acid aminotransferase; 4: Alanine aminotransferase; 5: Aspartate aminotransferase; 6: Ornithine aminotransferase; 7: Ornithine decarboxylase; 8: Glutamate decarboxylase; 9: Aspartate decarboxylase; 10: Histidine decarboxylase; 11: Carnosine synthase; 12: Carnosine N-methyltransferase

There is a large body of evidence showing heart-protective effects of carnosine and related HRCs (anserine and homocarnosine). Recent human study demonstrated that oral administration of carnosine increased exercise tolerance and quality of life in chronic heart failure patients [18]. Many animal studies showed that carnosine and HRCs protected heart tissue from ischaemia-reperfusion damage and cardiomyopathy [19–25], improved contractility of heart in cardiac ischemia [19,26], and decreased cardiotoxicity [24,27]. Carnosine has been reported to have several biochemical functions, including control of pH, antioxidant, metal-ion chelator, carbonyl scavenger, antiglycator, vascular tone

regulator, immunostimulant, and wound healing agent [28,29]. Owning to their imidazole group, HRCs have the crucial physiological function of proton or pH buffering [30]. This crucial function makes HRCs important to skeletal muscle, where there is highly expression of HRCs. Under anaerobic exercise, there is high production of lactic acid, in which its dissociation results in production of protons that brings pH lower than normal levels, which leads to muscle contractile fatigue. Here is where HRCs play a crucial role in proton sequestering to maintain physiological pH. Thus, it can be expected that HRCs also serve this function in heart muscle. In addition to acid-buffering activity, antioxidant activity is another important function of HRCs that have been reported to potentiate preventive effects on cardiac hypertrophy, heart failure, ischemia-reperfusion injury, and myocardial infarction [26,31,32]. Several *in vitro* and animal studies have demonstrated the ability of carnosine and HRCs to deactivate precursors of advanced lipoxidation end-products (ALEs) and advanced glycoxidation end-products (AGEs), leading to the inhibition of AGEs and ALEs formations [33]. Since ALEs and AGEs are associated in the onset and propagation of several oxidative-based diseases such as atherosclerosis, a key factor leading to heart disease, HRCs may exert heart-protective effects through this mechanism. Finally, based on several studies reporting antihypertensive, vasorelaxant, and intracellular calcium-regulating effects of HRCs, it can be expected that HRCs may play a role in modulating contraction/ relaxation of heart muscle [34–41].

Histamine have been reported to be inflammatory mediator and associated with atherosclerosis and myocardial infarction [42]. However, several recent studies have showed beneficial effects on heart. It was found to exert anti-inflammatory effect on human white blood cells [43]. It was also reported to exert a protective role in coronary arteries or heart blood vessels [44]. The study in mice lack of histidine decarboxylase demonstrated that histamine deficiency reduced heart function and enhanced the damage of infarcted heart. The study has suggested a protective role of histamine in the process of myocardial infarction through inhibition of cardiomyocyte apoptosis and promotion of macrophage infiltration that contributes to myocardial healing [45]. It has been proposed that in cardiovascular system, mast cells might not be the predominant source of histamine. Carnosine may serve as a non-mast cell reservoir for histidine as a substrate for histamine synthesis during stress conditions [45–47]. These results may suggest the importance of HRCs in heart.

GABA has been reported to be involved in the regulation of cardiovascular function in mammals, including humans [48]. It was found to exhibit regulatory effects on blood pressure and heart rate [48]. In inducible myocardial ischemia patients, a strikingly decrease in plasma levels of GABA was observed [49]. This compound has been reported to not only be present exclusively in the central nervous system in humans, but also be formed and present in other tissues such as kidney [50]. Based on these results, it is possible to consider that GABA may also play a role in heart protection.

Collectively, among complex metabolic pathways in heart, HRCs such as carnosine, anserine, homocarnosine, and histamine as well as GABA may be the key mediators taking important part in the regulation of heart function and cardiovascular system.

5. Conclusion

Over 60 years past of the discovery of the association of vitamin B6 in heart disease, we still have no complete understanding of its mechanisms. Although its preventive role in heart disease has been mostly addressed through homocysteine and inflammation hypotheses, many recent studies have demonstrated the controversial results and suggested that vitamin B6 may exert a heart-protective effect through other mechanisms. In the present study, we found that the vitamin B6 supplemental level (the 35 mg PN HCl/kg diet) increased the final metabolites in histidine metabolic pathways, which are carnosine, anserine, homocarnosine, and histamine in rat heart tissues, as compared with the marginal deficient level (the 1 mg PN HCl/kg diet). In the present chapter, we propose a novel putative mechanism underlying the heart-protective role of vitamin B6, by which vitamin B6 controls enzymes that are a rate-limiting step in the formation of β-alanine and GABA, which are the rate-limiting precursors of HRCs. Being reported to exert heart-protective effects, those HRCs may, in turn, play important roles in preventing heart disease. Although vitamin B6 is involved in a very large number of physiologic reactions and may affect heart disease in a complex fashion of inter-relationships among several factors and thereby it could be very hard to define the exact mechanism(s), our proposed novel mechanism may add a new piece in the puzzle of how vitamin B6 prevents heart disease.

References

[1] Cui R, Iso H, Date C, Kikuchi S, Tamakoshi A. Dietary folate and vitamin B6 and B12 intake in relation to mortality from cardiovascular diseases: Japan collaborative cohort study. *Stroke*. 2010; 41(6):1285-1289.

[2] Friso S, Lotto V, Corrocher R, Choi SW. Vitamin B6 and cardiovascular disease. In: Stanger O, editors. Water Soluble Vitamins. Netherlands: Springer; 2012. P. 265-290.

[3] Dhalla NS, Takeda S, Elimban V. Mechanisms of the beneficial effects of vitamin B6 and pyridoxal 5-phosphate on cardiac performance in ischemic heart disease. *Clin Chem Lab Med*. 2013; 51(3):535-543.

[4] Ntaios G, Savopoulos C, Grekas D, Hatzitolios A. The controversial role of B-vitamins in cardiovascular risk: An update. *Arch Cardiovasc Dis*. 2009; 102(12):847-854.

[5] Suidasari S, Hasegawa T, Yanaka N, Kato N. Dietary supplemental vitamin B6 increases carnosine and anserine concentrations in the heart of rats. *Springerplus*. 2015; 4(1):280.
[6] Rinehart JF, Greenberg LD. Arteriosclerotic lesions in pyridoxine-deficient monkeys. *Am J Pathol.* 1949; 25(3):481-491.
[7] McCully KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. *Am J Pathol*. 1969; 56(1):111-128.
[8] Maxwell SR. Coronary artery disease-free radical damage, antioxidant protection and the role of homocysteine. *Basic Res Cardiol*. 2000; 95 Suppl 1:I65-I71.
[9] Shen J, Lai C, Mattei J, Ordovas JM, Tucker KL. Association of vitamin B-6 status with inflammation, oxidative stress, and chronic inflammatory conditions: the Boston Puerto Rican Health Study. *Am J Clin Nutr*. 2010; 91:337-342.
[10] Friso S, Jacques PF, Wilson PWF, Rosenberg IH, Selhub J. Low Circulating Vitamin B6 Is Associated with Elevation of the Inflammation Marker C-Reactive Protein Independently of Plasma Homocysteine Levels. *Circulation*. 2001; 103(23):2788-2791.
[11] Folsom AR, Desvarieux M, Nieto FJ, Boland LL, Ballantyne CM, Chambless LE. B vitamin status and inflammatory markers. *Atherosclerosis*. 2003; 169(1):169-174.
[12] Lotto V, Choi S-W, Friso S. Vitamin B6: a challenging link between nutrition and inflammation in CVD. *Br J Nutr*. 2011; 106(2):183-195.
[13] Ueland PM, McCann A, Midttun O, Ulvik A. Inflammation, vitamin B6 and related pathways. *Mol Aspects Med*. 2016; 53:10-27.
[14] Blancquaert L, Baba S, Kwiatkowski S, et al. Carnosine and anserine homeostasis in skeletal muscle and heart is controlled by β-alanine transamination. *J Physiol*. 2016; 594(17):4849-4863.
[15] Petroff OAC. GABA and glutamate in the human brain. *Neuroscientist*. 2002; 8(6):562-573.
[16] Cronan JE. β-Alanine Synthesis in Escherichia coli. *J Bacteriol*. 1980; 141(3):1291-1297.
[17] Mueller SG, Weber OM, Boesiger P, Wieser HG. Influence of pyridoxal 5'-phosphate alone and in combination with vigabatrin on brain GABA measured by 1H-NMR-spectroscopy. *Brain Res Bull*. 2001; 55(4):555-560.
[18] Lombardi C, Carubelli V, Lazzarini V, et al. Effects of oral administration of orodispersible levo-carnosine on quality of life and exercise performance in patients with chronic heart failure. *Nutrition*. 2015; 31(1):72-78.
[19] Stvolinsky SL, Dobrota D. Anti-ischemic activity of carnosine. *Biochem*. 2000; 65(7):849-855.
[20] Lee JW, Miyawaki H, Bobst E V, Hester JD, Ashraf M, Bobst AM. Improved functional recovery of ischemic rat hearts due to singlet oxygen scavengers histidine and carnosine. *J Mol Cell Cardiol*. 1999; 31(1):113-121.
[21] Alabovsky VV, Boldyrev AA, Vinokurov AA, Shchavratsky VK. Effect of histidine-containing dipeptides on isolated heart under ischemia/reperfusion. *Biochemistry (Moscow)*. 1997; 62(1):77-87.
[22] Prokop'eva VD, Laptev BI, Afanas'ev SA. The protective effect of carnosine in hypoxia and reoxygenation of the isolated rat heart. *Biokhimiia (Moscow, Russia)*. 1992;57(9):1389-1392.
[23] Ozdoğan K, Taşkın E, Dursun N. Protective effect of carnosine on adriamycin-induced oxidative heart damage in rats. *Anadolu Kardiyol Derg*. 2011; 1:3-10.
[24] Zieba R, Wagrowska-Danilewicz M. Influence of carnosine on the cardiotoxicity of doxorubicin in rabbits. *Pol J Pharmacol*. 2003; 55(6):1079-1087.
[25] Bokeriya LA, Boldyrev AA, Movsesyan RR, et al. Cardioprotective effect of histidine-containing dipeptides in pharmacological cold cardioplegia. *Bull Exp Biol Med*. 2008; 145(3):323-327.
[26] Zhao Y, Zhao B. Protective effect of natural antioxidants on heart against ischemia-reperfusion damage. *Curr Pharm Biotechnol*. 2010; 11(8):868-874.
[27] Dursun N, TaşkIn E, Öztürk F. Protection against adriamycin-induced cardiomyopathy by carnosine in rats: Role of endogenous antioxidants. *Biol Trace Elem Res*. 2011; 143(1):412-424.
[28] Boldyrev AA, Aldini G, Derave W. Physiology and pathophysiology of carnosine. *Physiol Rev*. 2013; 93(4):1803-1845.
[29] Hipkiss AR. Carnosine and its possible roles in nutrition and health. *Adv Food Nutr Res.* 2009; 31:87-154.
[30] Abe H. Role of histidine-related compounds as intracellular proton buffering constituents in vertebrate muscle. *Biochemistry (Moscow)*. 2000; 65(7):757-765.
[31] Seddon M, Looi YH, Shah AM. Oxidative stress and redox signalling in cardiac hypertrophy and heart failure. *Heart*. 2007; 93(8):903-907.
[32] Evran B, Karpuzoğlu H, Develi S, et al. Effects of carnosine on prooxidant-antioxidant status in heart tissue, plasma and erythrocytes of rats with isoproterenol-induced myocardial infarction. *Pharmacol Reports*. 2014; 66(1):81-86.
[33] Vistoli G, Carini M, Aldini G. Transforming dietary peptides in promising lead compounds: The case of bioavailable carnosine analogs. *Amino Acids*. 2012; 43(1):111-126.
[34] Batrukova MA, Rubtsov AM. Histidine-containing dipeptides as endogenous regulators of the activity of sarcoplasmic reticulum Ca-release channels. *Biochim Biophys Acta-Biomembr*. 1997; 1324(1):142-150.
[35] Roberts PR, Zaloga GP. Cardiovascular Effects of Carnosine. *Biochemistry (Moscow)*. 2000; 65(7):856-861.
[36] Zaloga GP, Roberts PR, Nelson TE. Carnosine: a novel peptide regulator of intracellular calcium and contractility in cardiac muscle. *New Horiz*. 1996; 4(1):26-35.
[37] Zaloga GP, Roberts PR, Black KW, et al. Carnosine is a novel peptide modulator of intracellular calcium and contractility in cardiac cells. *Am J Physiol*. 1997; 272(1):H462-H468.
[38] Du Vigneaud V, Hunt M. The synthesis of D-carnosine, the enantiomorph of the naturally occurring form, and a study of its depressor effect on the blood pressure. *J Biol Chem*. 1936; 115:93-100.
[39] Niijima A, Okui T, Matsumura Y, et al. Effects of L- carnosine on renal sympathetic nerve activity and DOCA- salt hypertension in rats. *Auton Neurosci Basic Clin*. 2002; 97:99-102.
[40] O'Dowd A, O'Dowd J, Miller D. The dipeptide carnosine constricts rabbit saphenous vein as a zinc complex apparently via a serotonergic receptor. *J Physiol*. 1996; 495(2):535-543.
[41] Ririe DG, Roberts PR, Shouse MN, Zaloga GP. Vasodilatory actions of the dietary peptide carnosine. *Nutrition*. 2000;

16(3):168-172.
[42] Clemetson CAB. The key role of histamine in the development of atherosclerosis and coronary heart disease. *Med Hypotheses*. 1999; 52(1):1-8.
[43] Østerud B, Olsen JO. Pro- and anti-inflammatory effects of histamine on tissue factor and TNFα expression in monocytes of human blood. *Thromb Res*. 2014; 133(3):477-480.
[44] Chan CK, Liao SY, Zhang YL, Xu A, Tse HF, Vanhoutte PM. Protective effects of histamine on G_q-mediated relaxation in regenerated endothelium. *Am J Physiol Heart Circ Physiol*. 2014; 306(2):286-290.
[45] Deng L, Hong T, Lin J, et al. Histamine deficiency exacerbates myocardial injury in acute myocardial infarction through impaired macrophage infiltration and increased cardiomyocyte apoptosis. *Sci Rep. 2015; 5:13131.*
[46] Bae ON, Majid A. Role of histidine/histamine in carnosine-induced neuroprotection during ischemic brain damage. *Brain Res*. 2013; 1527:246-254.
[47] Flancbaum L, Fitzpatrick JC, Brotman DN, Marcoux AM, Kasziba E, Fisher H. The presence and significance of carnosine in histamine-containing tissues of several mammalian species. *Agents Actions*. 1990; 31(3/4):190-196.
[48] DeFeudis FV. γ-Aminobutyric acid and cardiovascular function. *Cell Mol Life Sci.* 1983; 39(8):845-849.
[49] Sabatine MS, Liu E, Morrow DA, et al. Metabolomic identification of novel biomarkers of myocardial ischemia. *Circulation*. 2005; 112(25):3868-3875.
[50] Zachmann M, Tocci P, Nyhan WL. The occurrence of gamma-aminobutyric acid in human tissues other than brain. *J Biol Chem*. 1966; 241(6):1355-1358.