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Review

# Biochemical pathways generating post-mortem volatile compounds co-detected during forensic ethanol analyses

Vassiliki A. Boumba *, Kallirroe S. Ziavrou, Theodore Vougiouklakis

*Department of Forensic Medicine and Toxicology, Medical School, University of Ioannina, 45110 Ioannina, Greece*

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**Abstract**

In this contribution are presented the fermentations of the main substrates present in a decaying corpse, namely carbohydrates, amino acids, glycerol and fatty acids, generating the post-mortem volatile compounds that could be detected along with ethanol during the forensic ethanol analysis. The available literature (preferably reviews) on microbial metabolic pathways (enzymes, substrates, conditions) that are implicated in the formation of these volatiles has been reviewed. The microbial formation of the following volatiles is supported by the presented biochemical data: ethanol, acetaldehyde, acetone, 2-propanol, 1-propanol, 1-butanol, isobutanol, isoamyl alcohol, *d*-amyl alcohol, acetate, propionate, butyrate, isobutyrate and ethyl esters (mainly ethyl acetate). The extracted information was correlated with the existing forensic literature on the post-mortem detected volatiles. The significance of the microbial produced volatiles on the selection of an appropriate internal standard for the ethanol analysis has been considered. Finally, the possible contribution of the presence of volatiles in the interpretation of ethanol analysis results in post-mortem cases is discussed.


*Keywords:* Ethanol; Propanol; Butanol; Fatty acids; Acetone; Alcohol; Post-mortem; Fermentation; Internal standard; Volatiles

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* Corresponding author. Tel.: +30 26510 97724; fax: +30 26510 97857.
*E-mail address:* vboumba@cc.uoi.gr (V.A. Boumba).

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## 1. Introduction

In the field of forensic toxicology, it is well recognized that the analysis of ethanol offers an intriguing challenge for the forensic toxicologist due to the possibility of its post-mortem formation and loss. There is significant evidence in the literature that ethanol is produced by microorganisms in animal and human corpse as well as in isolated tissues containing microbial burden after they have been stored for a few days at room temperature [1–21]. The amount of ethanol formed during the post-mortem interval is considered to depend on the species of microorganisms present, the availability of substrates, the ante-mortem conditions of the deceased and the storage condition of the body prior to collection of specimen for toxicological analysis [1]. It is worth mentioning, however, that ethanol is not found in all putrefied bodies [2,22,23]. This observation should be considered normally the result of the decrease of ethanol levels after an initial increase most possibly due to ethanol utilization by microorganisms [2,24].

The available substrates post-mortem that could give rise to microbial ethanol formation show a wide variety. There are included substrates, such as carbohydrates, amino acids and lipids [2,6]. During adipocere formation by bacteria glycerol is produced from fat hydrolysis and this compound has been suggested to be a potential substrate for post-mortem ethanol production [2].

So far glucose has been assumed to be the primary substrate for ethanol production during putrefaction of a corpse [2,25,26]. A probable reason for this assumption should be the fact that ethanol fermentation to produce alcoholic drinks always requires sugar (sucrose, glucose or fructose). On the other hand, glucose levels in blood or other tissues after death declines rapidly [2], whereas ethanol formation ceases after several days from death. We are of opinion that although glucose might indeed be the most readily utilized substrate by microbes, many other abundant compounds (amino acids, lactate, fatty acids, etc.) available in the corpse could serve as substrates for microbial growth. As a result of the microbial activity ethanol and other volatile compounds could be produced. However, the available information on the biochemical processes involving the substrates available post-mortem is sparse.

In this contribution, the authors present the microbial biochemical pathways generating the volatile compounds that have been reported to be detected in post-mortem cases during the forensic ethanol analysis. The available literature (preferably reviews) on microbial metabolic pathways (enzymes, substrates, conditions) that are implicated in the formation of these volatiles has been reviewed. The extracted information was correlated with the existing forensic literature on the post-mortem detected volatiles and the microbes implicated. Sources for this review were reports on fermentative pathways derived from the food industry (milk products, wine products, etc.) and for biochemical and microbiological processes in the bioreactors technology field. The leading idea for writing this review was, firstly, the lack of literature in the field of forensic toxicology to support the microbial neo-formation of the various volatiles that had been co-detected with ethanol during ethanol analysis from the post-mortem available substrates resting on biochemical data. Secondly, the authors' belief that a human corpse for the microbes serves just as a mixture of organic compounds which is the nutrient source for their growth. Subsequently, the biochemical functions in this system (although it is a complicated biochemical system) are similar with any other comprising substrates and microbes under the favorable conditions for microbial growth.

## 2. Putrefaction procedure

Under natural conditions the human body commences to decompose immediately after death. Decomposition is a complicated process that may differ from body to body, from environment to environment and even from one part of the same corpse to another. It includes several subclasses, which may merge into each other or may be uniform throughout the same cadaver [27].

Putrefaction is characterized by the progressive break down of soft tissues and the alteration of their protein, carbohydrate and fat constituents: it is due essentially to the actions of many enzymes, some of which are already present in an active or a latent form in the tissues, while others are derived from bacteria and fungi from the intestine and outer environment and sometimes from insects which may mature or are in a larval stage [27].

Shortly after death, microorganisms from the intestines migrate into the local tissues and gain access to lymphatic, blood-capillaries and veins and subsequently by way of tissue planes and the blood and lymph, to the body tissues generally. In many instances, a similar bacterial invasion commences from the respiratory system. This invasion occurs rapidly, especially at ambient and elevated temperatures. Aerobic organisms deplete the tissues of oxygen and although their numbers are reduced as the available oxygen diminishes, they create favorable chemical conditions for the more destructive anaerobic organisms which are derived mostly from the intestinal canal. In the later stages of decomposition some anaerobic species may come from soil or from the air. The bacterial flora thus changes from the aerobic groups, mainly

coliform–staphylococcal–proteus species, to the anaerobic population in which the clostridia predominate [27].

There is remarkable decrease in blood pH immediately after death [28]. Lack of circulating blood causes oxygen insufficiency within a short time, so that oxidative respiration ceases and conditions become anaerobic. Microbial enzymes hydrolyze polymers (lipids, carbohydrates and proteins) converting them into monomers (fatty acids, glycerol, monosaccharides and amino acids). As a result, the pH of blood steadily increases, and odorous compounds and gases (e.g. alkyl sulfides and methane) are produced along with ethanol and many other volatile compounds [1,5,6,8,19,28–30]. In general, the pH and the redox potential of tissues post-mortem affect the type of microorganisms able to grow, as well as their rate of growth [2].

Many species of bacteria, yeast and fungi have been shown capable of producing ethanol post-mortem [1,2]. The following bacteria species have been reported as the major colonizers in corpses and, in parallel, the main ethanol producers: *Clostridium perfringens* and other *Clostridium* species, enterobacteria (particularly, *Escherichia coli*, *Klebsiella* and *Proteus* species), micrococcaceae (mainly *Staphylococcus aureus*), streptococci (mainly *Streptococcus faecalis* and non-haemolytic streptococci) and *Bacillus* species (mainly *Bacillus subtilis*) [2]. Yeast capable of producing ethanol might also be found in decaying corpses but to a lesser extent (*Candida albicans*, other *Candida* species, *Saccharomyces cerevisiae* and *Saccharomyces* species, etc.) [2,18,31]. The greatest increases in ethanol levels have been usually attributed to enterococci or enteric bacilli [2,32].

## 3. Microbial metabolism under anaerobic conditions

Most microbes are heterothophs, capable of subsisting on a variety of carbon substrates, including carbohydrates, lipids, glycolipids, and dicarboxylic and amino acids. Carbon metabolism provides the bacterial cell with energy in the form of reducing equivalents and adenosine triphosphate (ATP), as well as essential biosynthetic precursors [33].

In their natural environment, microorganisms often encounter situations that change frequently and rapidly with respect to temperature, pH, oxygen and nutrient availability. In cases where mixtures of different substrates and energy sources are available, many organisms utilize one substrate preferentially and consume the additional substrates only when the preferred one is exhausted. A variety of coordinated and adaptive mechanisms and regulatory circuits have been evolved by microbes in order to adjust their physiology to allow their optimal growth. This adaptation is often mediated by substrate-specific induction or repression of catabolic genes [34–42].

From a microbiological point of view the chemical composition, physical form and amount of substrate available affects bacterial metabolism, which are also dependent on the types and numbers of different bacterial populations, catabolite regulatory mechanisms [34], the availability of inorganic electron donors, such as nitrate [35] and sulfate [36], as well as competitive and cooperative interactions between different species in the micro biota [37,38].

Under anaerobic conditions, microbes conduct a specific type of heterotrophic metabolism that is called fermentation [33]. Fermentation uses organic carbon as a terminal electron acceptor instead of oxygen or inorganic anions that are used during respiratory metabolism. This means that during fermentation is not used an electron transport chain to oxidize NADH (reduced nicotinamide adenine dinucleotide) to $NAD^+$ (oxidized nicotinamide adenine dinucleotide). Therefore, an alternative way of balancing this reducing power (NADH) and maintaining a supply of $NAD^+$ needed for other metabolic processes (e.g. glycolysis) should exist.

Fermentative organisms usually do not operate a complete tricarboxylic acid cycle to avoid overproduction of NADH [41]. A variety of oxidation–reduction reactions is involved in fermentative processes which are usually coupled to substrate level phosphorylation in order to produce energy (ATP). In substrate-level phosphorylation reactions an organic compound derived from the original fermentation substrate is phosphorylated to produce a high-energy organic compound (generally in the form of a CoA-ester). The phosphate group from the high-energy phosphate-containing organic compound is transferred to adenine diphosphate (ADP) to form ATP. During this process, NADH and other cofactors (ferredoxins, flavins) are used to produce many different reduced metabolic by-products and to regenerate $NAD^+$ required for glycolysis. These reduced organic compounds are generally small organic acids and alcohols (volatiles) derived usually from pyruvate, the end product of glycolysis.

Substrate-level phosphorylation reactions are energetically inefficient when compared with oxidative metabolism and they result in relatively low ATP yields. Large amounts of substrate are therefore required for growth of fermentative microorganisms, leading to the formation of substantial amounts of metabolic end products [39,41]. Fermentation reactions must be self-balancing with respect to the formation and consumption of reducing power, with the redox difference between substrates and end products determining the amount of energy that can be produced [40,41].

To a large extent, this process affects the flow of carbon through the bacteria, the energy yield that can be obtained from the substrate, and the fermentation products that are ultimately generated [34–41]. Synthesis of reduced products including hydrogen, lactate, succinate, butyrate and ethanol is used to keep the redox balance during fermentation, whereas the formation of more oxidized products, such as acetate, is associated with ATP production. Conversely, when more reduced fermentation products are formed, this process results in comparatively low ATP yields [41]. Many anaerobes take advantage of the flexibility offered by branched fermentation pathways, which allow them to adapt the thermodynamic efficiency of substrate catabolism, in response to changing environmental conditions, through modulating ATP formation and redox balance. Key control molecules in the microbial metabolism are the metabolites pyruvate and acetyl-CoA, which can be converted into a wide range of products.

The relationships between fermentative organisms are such that the products of one group of organisms can serve as

substrates for another group of organisms [33,36,38]. In other words, the various organisms are lined up with their catabolic activities and form the so-called ''anaerobic food chain''. In this chain, the growth of acitogenic and acetogenic bacteria [42] precedes that of methanogenic bacteria [43].

Fermentations are carried out by facultative or obligatory anaerobic bacteria or yeasts (anaerobes). Most commonly they are classified according to the main end product of the process (e.g. ethanol fermentation, lactate fermentation) [33].

## 4. Carbohydrates as microbial substrates

Carbohydrates and especially hexoses, such as glucose, are the preferred carbon and energy sources for many microbes. Microbes possess two mechanisms for carbohydrate catabolism: the Embden–Meyerhof–Parnas (EMP) glycolytic pathway and the Entner–Doudoroff (ED) pathway [33]. The end product of these glycolytic processes is pyruvate.

The most commonly used pathway for glucose uptake and conversion to pyruvate is the EMP glycolytic pathway [33]. This occurs in animals, plants, many bacteria and yeasts. The sum of the reactions generating two molecules of pyruvate and ATP per glucose molecule is as follows (Pi: inorganic orthophosphate):

$$\text{Glucose} + 2\,\text{ADP} + 2\,\text{NAD}^+ + 2\,\text{Pi} \rightarrow 2\,\text{Pyruvate} + 2\,\text{ATP} + 2\,\text{NADH} + 2\text{H}^+.$$

The ED pathway, which is absent in mammals, is now considered to be fairly widespread particularly among Gram-negative bacteria, while it is not very common in anaerobes [33,44]. The ED pathway yields only one ATP per two pyruvate molecules.

The ability of microbes to produce ethanol from glucose is widespread and most available information concerns this substrate. Many species form ethanol as a major end product of their metabolism as it is a highly reduced metabolite.

### 4.1. Ethanol fermentation by yeasts and bacteria

Ethanol originates from the fermentative breakdown of glucose and other hexoses by several yeasts, bacteria and fungi [45,46]. Yeasts grow only for some generations under anaerobic conditions since they are not truly facultative anaerobic microbes [33]. *S. cerevisiae* is considered the main ethanol producing yeast specie [47].

Glucose degradation in yeasts is employed via the EMP pathway. Pyruvate is decarboxylated by pyruvate decarboxylase to acetaldehyde which is then reduced to ethanol by alcohol dehydrogenase [33,47]. The reactions are schematically shown in Fig. 1, pathway A.

Ethanol fermentation of glucose and other carbohydrates could also be carried out by bacteria species [45,48]. For some of them the conversion of glucose to pyruvate is achieved via the ED pathway (as for *Zymomonas* sp.) [49] while for others via the EMP pathway [45,48]. The final conversion of pyruvate to ethanol involves the enzymes pyruvate decarboxylase and

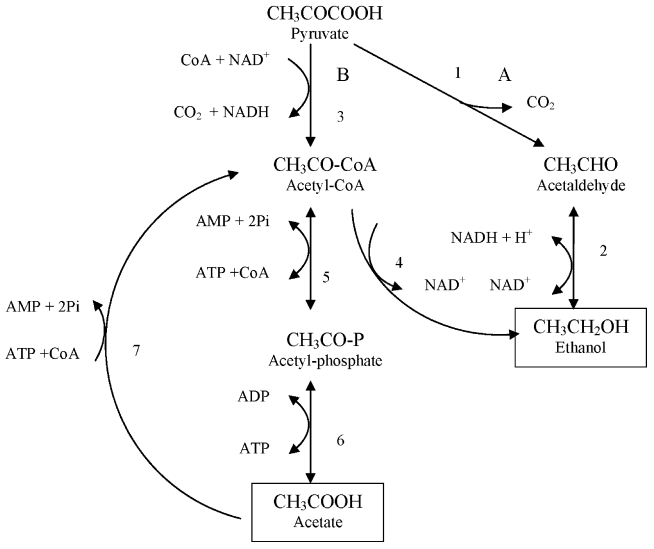


Fig. 1. Fermentation of glucose to ethanol by yeasts and bacteria species. The reactions shown are from the stage of pyruvate to the final products. (A) Yeasts and some bacteria species (see text): 1, pyruvate decarboxylase; 2, alcohol dehydrogenase; (B) bacteria: 3, pyruvate dehydrogenase or pyruvate-formate lyase; 4, acetaldehyde/alcohol dehydrogenase; 5, phosphotransacetylase; 6, acetate kinase; 7, AMP-forming acetyl-CoA synthetase [53].

alcohol dehydrogenase as it is for yeast (Fig. 1A). This fermentative pathway is carried on by species such as *Zymomonas mobilis*, *Zymomonas anaerobica* [49], *Sarcina ventriculi* and *Erminia amylovora* [33,45,48].

Pyruvate decarboxylase, however, is rare in bacteria [44,50]. For these bacteria, such as many lactic acid bacteria (*Streptococcus lactis*, *Leuconostoc mesenteroides*, etc.), enterobacteria (*E. coli*, *Klebsiella aerogenes*, etc.) and clostridia (*C. splenoides*, *C. sporogenes*, etc.) the formation of considerable amounts of ethanol from carbohydrates is achieved via acetyl-CoA [33,51,52]. Acetyl-CoA is a well recognized high-energy intermediate that sits at the crossroads of central metabolism [53]. The conversion of pyruvate to acetyl-CoA can occur oxidatively under aerobic conditions and non-oxidatively under anaerobic conditions. Oxidative decarboxylation is catalyzed by the pyruvate dehydrogenase complex. Non-oxidative decarboxylation of pyruvate to acetyl-CoA and formate is catalysed by the pyruvate-formate lyase system [53].

The sequence of reaction from pyruvate to ethanol is presented in Fig. 1, pathway B. The fate of acetyl-CoA can follow two alternative pathways: either conversion to acetate or reduction to ethanol. The conversion of acetyl-CoA to acetate is catalyzed by the phosphotransacetylase-acetate kinase system, generates ATP and does not consume reducing equivalents. The reduction of acetyl-CoA to ethanol is catalyzed by acetaldehyde/alcohol dehydrogenase, through the formation of acetaldehyde, and consumes reducing equivalents [53]. Therefore, bacteria can balance their requirements for reducing nucleotides and energy by modulating the amount of ethanol and acetate formed. The production of ethanol via acetyl-CoA results in addition to the formation of various other by-products (Sections 4.2 and 4.3).

### 4.2. Lactate fermentation

Some bacteria genera – often referred to as lactic acid bacteria (LAB) – produce lactic acid as a major metabolic end product of carbohydrate fermentations [54,55]. They are facultative anaerobes and have limited biosynthetic capacity. The comprising genera are *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Carnobacterium* and *Enterococcus*. LAB are classified according to the main hexose fermentation pathways they follow in two groups [33]. The homofermentative LAB catabolize glucose through the EMP pathway to pyruvate which is further reduced to lactic acid by lactate dehydrogenase.

The heterofermentative LAB convert glucose to mixed acid products (lactate, acetate, ethanol and carbon dioxide). In Fig. 2 are shown schematically the reactions involved in lactate fermentation. Pyruvate can either be converted to lactate by lactate dehydrogenase (LDH) [54] or to acetyl-CoA and $CO_2$ by pyruvate-formate lyase depending on whether the specific sugar uptake rate is high or low [56,57]. The subsequent reduction of acetyl-CoA by acetaldehyde and alcohol dehydrogenases yields as an end product ethanol. Acetate is also formed.

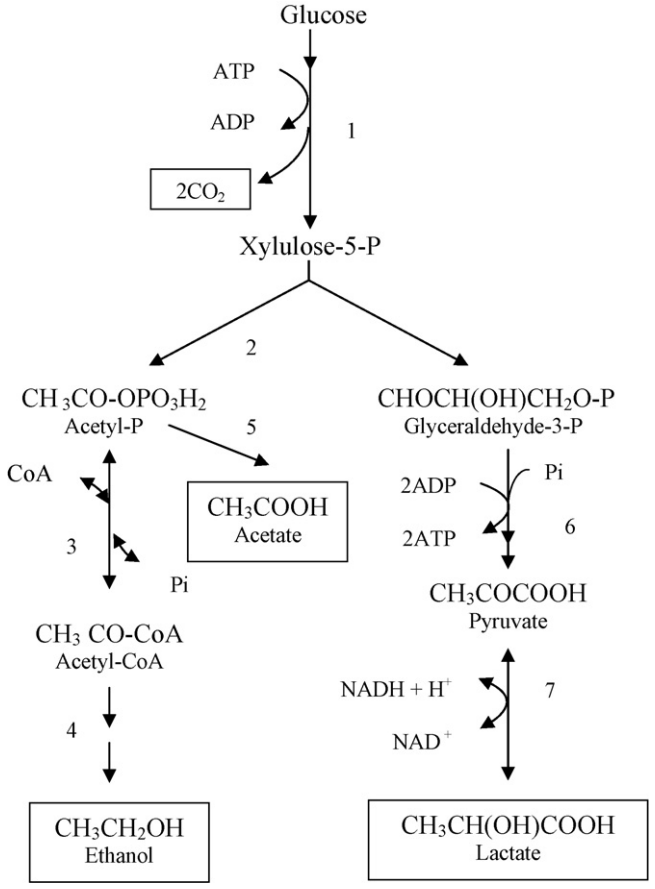


Fig. 2. Fermentation of glucose by LAB through the heterofermentative pathway to ethanol, lactate and carbon dioxide. 1, Hexokinase/glucose-6-phosphate dehydrogenase/6-phosphogluconate dehydrogenase/ribulose-5-phosphate-3-epimerase; 2, phosphoketolase (the cleavage reaction results finally in the formation of glyceraldehyde-3-phosphate and acetyl-phosphate) [33]; 3, phosphotransacetylase; 4, acetaldehyde/alcohol dehydrogenase; 5, acetate kinase; 6, enzymes of the Embden–Meyerhof–Parnas pathway [33]; 7, lactate dehydrogenase.

Lactate could be considered an important substrate for ethanol formation post-mortem since LDH and lactate are found in human tissues, as it has been previously suggested [6]. Actually, the conversion of lactate to pyruvate is feasible by the action of LDH (Fig. 2). Pyruvate can be further used for the formation of ethanol as it was already shown (Fig. 1).

Furthermore, lactate is a preferring substrate of the propionic acid bacteria which are anaerobic to aero-tolerant organisms [58]. The theoretical stoichiometry of propionic acid fermentation is as follows:

$$3\,\text{Lactate} \rightarrow 2\,\text{Propionate} + \text{Acetate} + CO_2 + H_2O.$$

Acetate could be converted through the action of acetate kinase or acetyl-CoA synthetase to acetyl-CoA giving rise to ethanol formation (Fig. 1) or could be accumulated in the medium [53]. The fate of propionate from the propionibacteria could generate 1-propanol [58] (Section 7).

### 4.3. Mixed acid and butanediol fermentation

This type of fermentation is carried out by the enterobacteria [33]. Species belonging to the genera *Escherichia*, *Salmonella* and *Shigella* ferment sugars to a mixture of products consisting of lactate, acetate, succinate and formate (mixed acid fermentation). In addition are formed $CO_2$, $H_2$ and ethanol (Fig. 3). Species of the genera *Enterobacter*, *Serratia* and *Erwinia* produce less acids, more gas ($CO_2$) and ethanol and above all large amounts of 2,3-butanediol (butanediol fermentation) [59].

Enterobacteria employ the EMP pathway for hexose breakdown [33]. In Fig. 3 are presented the biochemical pathways followed during the mixed acid and butanediol fermentations. Succinate results from phosphoenolpyruvate, the precursor of pyruvate (not shown). All the other end fermentative products (minus succinate) are derived from pyruvate. In the mixed acid fermentation large amounts of lactate are formed by the action of LDH. Little amount of lactate only is produced during the butanediol fermentation. The ratio of the fermentation products formed depends on the activity of the three-enzyme systems that could act on pyruvate. The first enzyme is LDH, responsible for the pyruvate conversion to lactate [54]. The second enzyme is the pyruvate-formate lyase system which yields formate and acetyl-CoA [60]. The third is α-acetolactate synthase which converts pyruvate to α-acetolactate [61].

The enterobacteria possess two enzyme systems for pyruvate conversion to acetyl-CoA. The first one is pyruvate dehydrogenase multi-enzyme complex which is involved in aerobic metabolism and generates two additional NADH per glucose while NADH inhibits its activity. Under anaerobic conditions, the enzyme present is inhibited by the high concentrations of NADH which cannot be deoxidized to $NAD^+$ and furthermore, it is no longer synthesized. Instead, under anaerobic conditions, the synthesis of pyruvate-formate lyase is induced. The advantage of the pyruvate-formate lyase over the

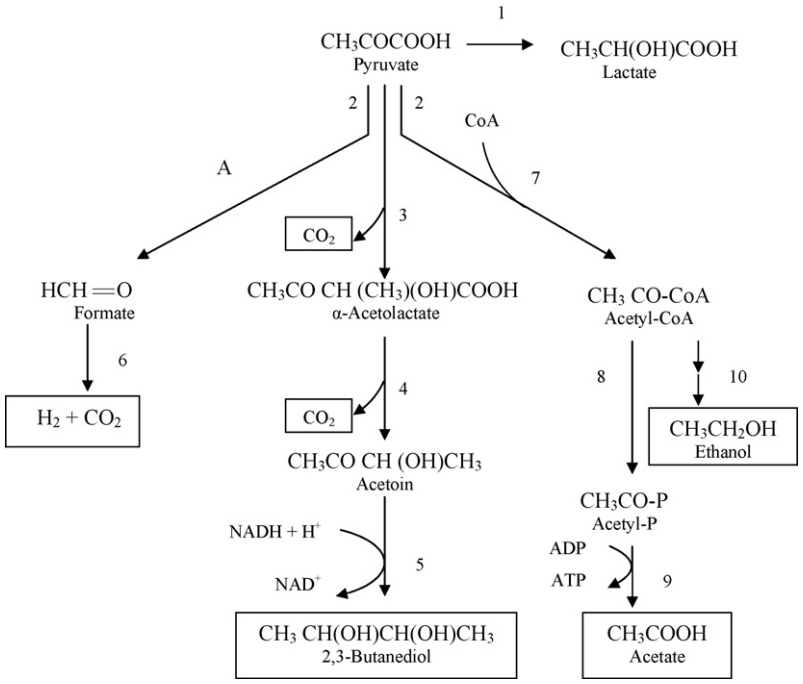


Fig. 3. Mixed acid and butanediol fermentation carried out by the enterobacteria. 1, Lactate dehydrogenase; 2, pyruvate-formate lyase; 3, formate-hydrogen lyase; 4, alpha-acetolactate synthase; 5, alpha-acetolactate decarboxylase; 6, 2,3-butanediol dehydrogenase; 7, phosphotransacetylase; 8, acetate kinase; 9, acetaldehyde/alcohol dehydrogenase.

pyruvate dehydrogenase complex in fermentative metabolism is that the formation of acetyl-CoA is not accompanied by the reduction of $NAD^+$ [53].

The enzyme alpha-acetolactate synthase is also responsible for 2,3-butanediol formation from pyruvate by bacilli [61]. This synthase is formed and is active under slightly acidic conditions (also referred to as the pH 6 enzyme).

### 4.4. Butyrate and butanol-acetone fermentation

Butyrate is formed as a main fermentation product by a number of obligate anaerobes belonging to the four genera *Clostridium*, *Butyrivibrio*, *Eubacterium* and *Fusobacterium* [52,62]. The clostridia employ the EMP pathway for degradation of hexoses to pyruvate [33].

The sequence of reactions involved in this type of fermentation is summarized in Fig. 4. Pyruvate is converted to acetyl-CoA by the enzyme system pyruvate-ferredoxin oxidoreductase. Acetyl-CoA is further converted to acetoacetyl-CoA whose fate can proceed through two branches either to acetone or to butyryl-CoA formation. Part of acetone could be reduced to 2-propanol (Section 7). Butyryl-CoA can be either converted to butyrate or reduced to 1-butanol. Butyrate is not formed from butyryl-CoA by simple hydrolysis; this would be a waste of energy. Instead butyryl-CoA is converted to butyryl-phosphate and finally the phosphate group is transferred to ADP. These reactions proceed under catalysis of the enzymes phosphotransbutyrylase and butyrate kinase, which are analogous to phosphotransacetylase and acetate kinase [33,52,62].

Some butyrate producing clostridia form simultaneously small amounts of 1-butanol [62]. With a few species, however, a real shift from butyrate production to solvent production (1-butanol and acetone or 2-propanol) can be observed under certain conditions. These species include *C. acetobutylicum* (the prominent one), *C. beijerinckii*, *C. tetanomorphum* and *C. aurantibutyricum* [52]. Since 1-butanol is very toxic (it is well known that microbes are not only sensitive to exogenous alcohols but also to those which are produced within the cell [63]) its final concentration is in the order of 2% (relatively low) [33].

It is worth mentioning that this type of fermentation proceeds with the parallel production of acetate, lactate and ethanol. Although the metabolic pathways have been described, the understanding of the mechanisms of their regulation is incomplete. As it usually happens in branched fermentations the flow down each branch is variable in response to various factors including pH, intracellular concentration of ATP and reduced pyridine nucleotides, nutrient limitation and the interplay between pathways or carbon and electron flow [52].

### 4.5. Aspects of the fermentations of carbohydrates

Glucose and other hexoses are the preferred carbon and energy source for many bacteria. The meaning of ‘‘the preferred substrate’’ is in general that it supports the best growth rate and/or growth yield [41]. In conditions where a mixture of different substrates are available instead of a single substrate, many organisms utilize one carbon source preferentially and consume the additional carbon sources only when the preferred one is exhausted or extremely limited [34,64]. This is the condition in the corpse during the putrefaction process.

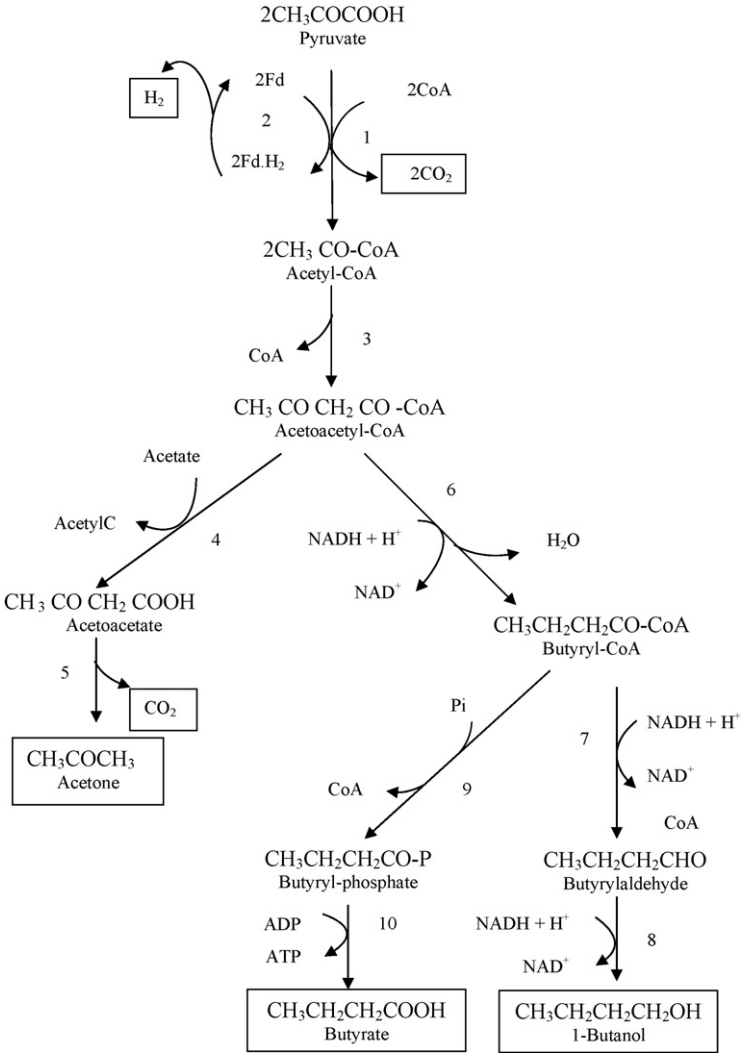


Fig. 4. Metabolic pathway for 1-butanol and acetone formation by clostridia. 1, Pyruvate-ferredoxin oxidoreductase; 2, hydrogenase; 3, acetyl-CoA-acetyltrasferase (thiolase); 4, acetoacetyl-CoA-acetate coenzyme A transferase; 5, acetoacetate decarboxylase; 6, L(+)-β-hydroxybutyryl-CoA dehydrogenase, crotonase and butyryl-CoA dehydrogenase; 7, butyraldehyde dehydrogenase; 8, butanol dehydrogenase; 9, phosphotransbutyrylase; 10, butyrate kinase.

The main purpose of sugar metabolism is to provide the bacterial cell with energy in the form of reducing equivalents and ATP, as well as essential biosynthetic precursors [41]. Most of the available carbohydrates could be converted to a mixture of fermentation products. The major soluble products are acetate and ethanol [34,38,65].

The production of acetate (acetogenesis) and ethanol results from the need to regenerate the $NAD^+$ consumed in glycolysis and to recycle coenzyme A (CoASH) required for the conversion of pyruvate to acetyl-CoA. The complete oxidation of acetyl-CoA to carbon dioxide occurs in the tricarboxylic acid cycle. Whenever this cycle does not operate completely (absence of oxygen or excess glucose) or the carbon flux into bacterial cells exceeds its capacity and that of other central metabolic pathways, acetate and ethanol are produced predominantly through mixed-acid fermentation procedures [41]. A number of environmental factors influence the composition of excreted products. One of the most important is the pH: near or above 7 the predominant products are acetate, ethanol and formate; as the pH drops cells produce more lactate than acetate and formate [53].

After death, carbohydrates are consumed firstly since they are the preferred substrate for many microbes. The carbohydrate fermentations, whenever the conditions are favorable for them to proceed, are generating the greatest portion of the post-mortem produced ethanol. This could explain the fact that ethanol synthesis is observed in the early stages of putrefaction. At this time can also be formed 1-butanol, acetone and 2-propanol, however, to a much lesser extent. If specimen from a corpse at this stage could be analyzed the volatile 1-butanol would probably be detected as a new product. The volatiles acetone and 2-propanol would be found elevated compared to their levels at death.

As carbohydrates are exhausted microbes start to consume other available substrates (amino acids, glycerol and fatty acids). Meanwhile, the micro flora in the corpse changes and the succession of microbes permits the growth of more destructive species such as the clostridia genera. As a

consequence the rate of ethanol synthesis declines and its levels remain relatively constant. There is also the possibility for the ethanol to be consumed by microbes. This could also result in volatile formation [66]. However, the consumption of ethanol by bacteria is a thermodynamically unfavorable reaction and proceeds only when acetate and hydrogen are consumed [67]. Consequently, it is not possible to proceed at this stage where there is availability of other substrates. Simultaneously, other volatiles such as the higher alcohols start to be produced in the putrefying corpse as a result of the fermentation of other substrates (presented in the following sections).

## 5. Amino acids as microbial substrates

Amino acids as microbial substrates generate the volatile compounds that could be found in the literature under the name higher alcohols or fusel alcohols. The term higher alcohols is used in the literature to define mainly 1-propanol, 2-methyl-propanol (isobutyl alcohol), 2-methyl-1-butanol (active- or *d*-amyl alcohol) and 3-methyl-1-butanol (isoamyl-alcohol). The mixture of these alcohols is also referred to as fusel alcohols since they are the principal components of the fusel oil which is formed during alcoholic fermentation by variable microorganisms. Moreover, the term ''branched-chain alcohols'' is used to define isobutanol, isoamyl and *d*-amyl alcohol since they result from the branched-chain amino acids valine, leucine and isoleucine, respectively.

### 5.1. Microbial formation of higher alcohols

Key intermediates for the higher alcohols formation are the respective alpha-ketoacids. These ketoacids could derive either catabolically from exogenous amino acids (''Ehrlich'' pathway) or could be the result of amino acid anabolic biosynthetic pathways (Fig. 5) [68]. The pathway choice depends on the individual higher alcohol and on the levels of available amino acids. The importance of the anabolic pathway decreases as the number of carbon atoms in the alcohol increases [68] and increases as amino acids are depleted [69].

As could be seen in Fig. 5, branched-chain α-ketoacids could also be synthesized *de novo* from carbohydrates [33,70]. Threonine is first deaminated by threonine deaminase to the α-ketobutyrate. Pyruvate can be converted with α-ketobutyrate to isoleucine, a reaction that is catalyzed by pyruvate acetohydroxy-acid synthase. This enzyme also plays a role in the conversion of two molecules of pyruvate to valine and leucine. The existing interrelation of the amino acid biosynthetic pathways could explain the fact that all higher alcohols (branched-chain alcohols and 1-propanol) are formed (in different quantitative patterns) during fermentative processes. As regards the formation of 1-propanol other metabolic processes from glycerol or from fatty acids could also proceed as well.

The metabolic route of higher alcohols formation from the deamination or transamination of extracellular threonine and branched-chain amino acids through the formation of the respective ketoacids is known as the ''Ehrlich pathway'' to honor the originator of the ideas [71,72].

The ''Ehrlich pathway'' is generally accepted to proceed in yeasts and other microbes. It involves an aminotransferase reaction which yields the corresponding α-ketoacids from the respective amino acids, a decarboxylation reaction which results in aldehyde formation, and finally, the reduction of aldehydes to the corresponding alcohols.

### 5.2. Higher alcohol formation by yeast

In recent studies performed with *S. cerevisiae*, the pathways of formation of isobutanol, *d*-amyl and isoamyl alcohols have been rather clearly established [73–75]. In the relative studies

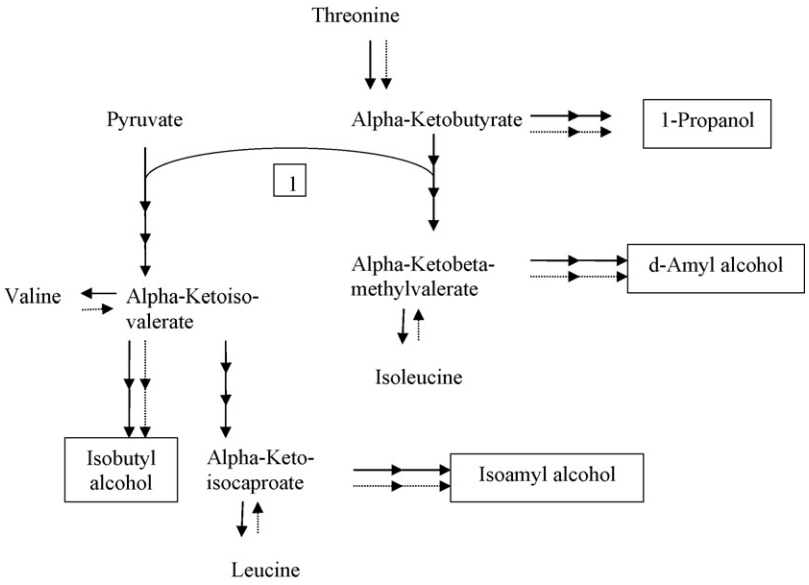


Fig. 5. Interrelation of amino acid metabolic pathways implicated in higher alcohol formation. Solid arrows, biosynthetic pathways; dotted arrows, Ehrlich pathways; 1, pyruvate acetohydroxy acid synthase.

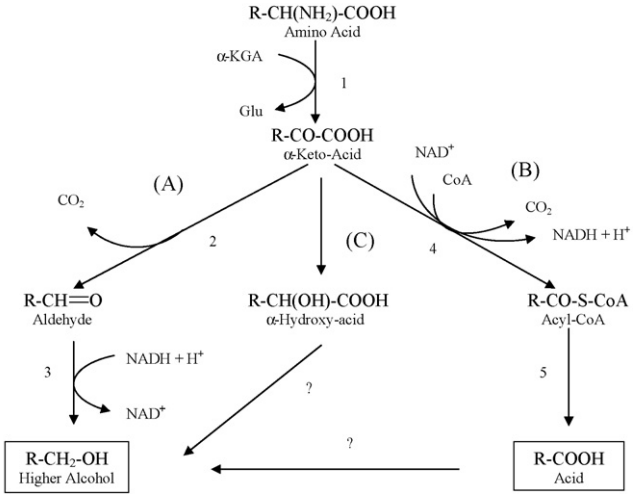


Fig. 6. Metabolic pathways for the metabolism of leucine, isoleucine, valine and threonine to isoamyl alcohol, *d*-amyl alcohol, isobutanol and 1-propanol, respectively, in yeasts and bacteria species. 1, Branched-chain amino acid amino transferase; 2, pyruvate decarboxylase; 3, alcohol dehydrogenase; 4, branched-chain α-keto-acid dehydrogenase; 5, acyl-CoA hydrolase; 6, reductase (speculated activity). R–: $CH_3CH(CH_3)$– for valine; $C_2H_5CH(CH_3)$– for isoleucine; $CH_3CH(CH_3)CH_2$– for α-ketobutyric acid which is the corresponding to threonine α-ketoacid. Threonine is firstly converted to the unstable product α-amino-2-butenoic acid by threonine dehydratase which is then deaminated by threonine deaminase to the α-ketobutyrate [33]. The α-ketoacids formed from the respective amino acids are α-ketoisovaleric acid from valine, α-keto-β-methyl-valeric acid from isoleucine, α-keto-isocaproic acid from leucine and α-ketobutyric acid from threonine.

were used radio-labeled, with $^{13}C$, leucine, valine and isoleucine, respectively, as the sole nitrogen source, in cultures of wild type strains of *S. cerevisiae*. The first step has been confirmed that is actually a transamination reaction of the respective branched-chain-amino acids to α-ketoacids (catalyzed by a cytosolic aminotransferase) [76]. The relevant results suggested three credible routes leading from α-ketoacids to higher alcohols [73–75].

The first route is the classical ‘‘Ehrlich pathway’’ which is schematically shown in Fig. 6, pathway A. The authors suggested that the decarboxylation of the α-ketoacids to aldehydes is performed by different enzymes as follows: α-ketoisocaproate by α-ketoisocaproate decarboxylase, α-ketoisovalerate and α-ketomethylvalerate by different isoenzymes of pyruvate decarboxylase [74,77]. The subsequent conversion of the aldehydes to the respective alcohols was suggested to be performed by alcohol dehydrogenases (isoenzymes of ethanol dehydrogenase and others).

The other two proposed potential metabolic routes presented in Fig. 6 (pathways B and C) implicated in the branched chain alcohol formation have been supported by the fact that in the relevant experiments have been detected all the $^{13}C$-labeled intermediate products. This possibility was further supported by the finding that the pyruvate decarboxylase activity (catalyzing the conversion of the α-ketoacid to aldehyde) was not essential for the fusel alcohol production by *S. cerevisiae* [78]. More specifically the second route was supported in *S. cerevisiae*, by the characterization of an enzyme complex with branched-chain α-ketoacid dehydrogenase activity (with unknown yet physiological role). This activity was found to catalyze the conversion of α-ketoisocaproate, α-ketomethylvalerate, and α-ketoisovalerate to isovaleryl-CoA, α-methylbutyryl-CoA and isobutyryl-CoA which are further converted to isovalerate, α-methylvalerate and isobutyrate, by acyl-CoA-hydrolase [79]. It was suggested that the acids formed could result to isoamyl alcohol, amyl alcohol and isobutanol, respectively, by the action of an unknown yet enzyme activity.

The third suggested route (pathway C) could be via a reductase that would form the respective α-hydroxyl-acids (Fig. 6, pathway C). However, these two routes (B and C) have not been precisely confirmed so far, either because the relative enzymes activities have not been detected yet (reductase), or because their true metabolic significance in yeasts is not yet apparent.

In yeasts α-ketobutyrate (the precursor of 1-propanol) is formed from threonine. Threonine is firstly converted by threonine dehydratase to the unstable product α-amino-2-butenoic acid and further deaminated by threonine deaminase to the α-ketobutyrate [33].

The ‘‘Ehrlich’s pathway’’ has been considered to be the predominant pathway for 1-propanol formation from its precursor α-ketobutyrate by yeast [80–84].

### 5.3. Higher ethanol formation by bacteria

Several data support the possibility of higher alcohols formation by bacteria from their relevant α-ketoacids by the same pathways followed by yeasts [82,84–89].

The study of Janssen [84] describes a *Clostridium* sp. able to produce 1-propanol from threonine and gives indications that the pathway B, presented in Fig. 6, for the metabolism of threonine to 1-propanol might also be operative in bacteria species. The author suggests that threonine dehydratase converts threonine to α-ketobutyrate, which is then oxidised to propionyl-CoA by α-ketobutyrate synthase. Part of the propionyl-CoA is metabolised further to propionate via an ATP-forming pathway and finally to 1-propanol. The author could not detect in the cultures activities responsible for 1-propanol formation – except for a possible weak activity of a NAD-linked propanol dehydrogenase – even though 1-propanol was obviously formed.

Studies performed with the Gram-negative bacilli of the genus *Zymomonas* have indicated that *Zymomonas* sp. and yeast share similar biosynthetic pathways for higher alcohol production [49,86]. These bacteria metabolize glucose through the ED pathway and show greater potential than the yeasts for the production of ethanol [49,80]. *Zymomonas* species have been used for the production of alcoholic beverages which contain several higher molecular weight alcohols as their main constituents, such as 1-propanol, isobutanol, isoamyl alcohol and *d*-amyl alcohol. Most of the work done with *Zymomonas* sp. indicated that the Ehrlich pathway is operative in *Zymomonas* sp. [86].

Further support of the possibility of similar pathways between yeasts and bacteria has been supported by the finding

of a branched-chain ketoacid dehydrogenase in many bacteria, including *Bacillus subtilis* [85], *Pseudomonas puties* [87], *Enterococcus faecalis* [88] and *Staphylococcus xylosus* [89]. Moreover, has been demonstrated the presence of β-methyl branched aldehydes in *S. xylosus* cultures during the growth phase [90]. Although the formation of the relative alcohols is not reported in this study, the possibility of their formation cannot be excluded since bacteria posses the enzyme alcohol dehydrogenase which is responsible for the alcohol formation from the respective aldehydes.

### 5.4. Ethanol formation from threonine

Another enzyme that could act upon threonine is threonine aldolase which has been characterized in yeast [91]. This enzyme transforms threonine into glycine and acetaldehyde. Acetaldehyde can be reduced to ethanol by alcohol dehydrogenase. The existence of this system could explain the transformation of threonine-$^{14}$C into labeled ethanol that was observed during alcoholic fermentations of different grain mashes [81].

### 5.5. Aspects of higher alcohol formation

In this section are commended some aspects of the higher alcohol formation that either are commonly reported in the relative studies reviewed or show particular interest for the post-mortem volatile production.

The production of higher alcohols from the respective α-ketoacids has been suggested to be an assimilation mechanism of the overflow of the respective amino acids, valine, leucine, isoleucine and threonine, four amino acids whose metabolism has several enzymic steps in common and is under the control of interrelated feedback mechanisms (Fig. 5). This suggestion was first made by Ehrlich [70] and confirmed later by others [76]. In yeast cultures branched-chain amino acids or alpha ketoacids have been used as the sole source of nitrogen and has been shown that actually they can serve this way [73–75,76]. Further support for this aspect was provided by the finding that other amino acids – expect valine, leucine, isoleucine and threonine – follow the same sequence of reaction in order to be converted to the corresponding alcohols when they are used as nitrogen source in the medium (e.g. methionine, phenylalanine) [92,93].

Indicative of the physiological significance of higher alcohol formation are the following two observations: firstly that the metabolism of glucose is needed for their production [81] and secondly that the appearance of fusel alcohol coincided with the formation of ethanol [81,82,94]. The explanation given to this aspect was that in the presence of glucose, more reductive coenzymes were available for the synthesis of higher alcohols from precursors, even though it is possible that some of the higher alcohols were being produced from glucose. Competition exists for the NADH, alcohol dehydrogenase, and the carboxylase thereby explaining the lag between the disappearance of the amino acids and the appearance of the higher alcohols observed in the relative studies [74–76,81]. The reductive formation of alcohols should be able to serve as a mechanism for recycling reduced cofactors during amino acid catabolism. Therefore, higher alcohol formation, although unusual, has been suggested to be a mechanism for disposal of electrons during amino acid fermentation [84,95].

Last but not least aspect is that although the quantitative pattern of higher alcohol formation is variable, in most cases 1-propanol predominates. A possible explanation that could be given to this aspect is that 1-propanol could result directly from amino acid sources (threonine) independently of the carbohydrate metabolism (needed for the other higher alcohols) and the glucose availability in the medium (Fig. 5). Another possibility is the formation of 1-propanol from other pathways (minus the ''Ehrlich pathway'') and substrates (minus amino acids). Actually, a deeper insight into the microbial metabolic pathways revealed more complex mechanisms for 1-propanol formation, some of which are not yet fully explained, as it would be presented in the next section concerning glycerol as substrate.

## 6. Glycerol as microbial substrate

Glycerol can be a product either from the hydrolysis of lipids or from carbohydrate metabolism [96]. On the other hand, glycerol can serve as a fermentation substrate for yeast as well as bacteria species. A number of yeasts can dissimilate glycerol, such as *S. cerevisiae*, *D. hansenii*, *Z. rouxii*, *C. glycerogenes* and *S. pombe* [96]. The bacterial species capable to ferment glycerol include enterobacteria of the genera *Klebsiella* (*K. pneumoniae*), *Enterobacter* (*E. agglomerans*) and *Citrobacter* (*C. freundii*), *Lactobacilli* (*L. brevis* and *L. buchneri*), clostridia of the *C. butyricum* and the *C. pasterianum* group [97] and propionibacteria [98].

The relevant reports show that part of glycerol is fermented to the same products as in carbohydrate fermentations [97]. This conversion provides the necessary energy for growth but the releasing of reduced equivalents demands a process for their oxidation. The oxidation is achieved by a reductive conversion of another part of glycerol which produces propanediols, propionate and 1-propanol [98].

The first fermentation pathway has been extensively studied in yeasts while recent data suggest that it is operative in enterobacteria and clostridia [97]. In Fig. 7 are shown the fermentative reactions from glycerol to pyruvate and from pyruvate to ethanol and the other end products as in carbohydrate fermentation.

The reactions down to the stage of pyruvate are common to all organisms involved. The intermediates of this sequence of reactions, glycerol-3-phosphate and dehydroxyacetone, can serve as precursors in the synthesis of phospholipids and glycerolipids, while dehydroxyacetone is also a precursor for amino acid synthesis [99]. The fate of pyruvate is different [100]. In the enterobacteria, the same sequence of reactions as in butanediol and ethanol fermentation presented in Figs. 1 and 2, are followed. From acetyl-CoA, is formed acetic acid via acetyl-phosphate, yielding extra ATP as well as ethanol, involving two NADH-oxidising steps with acetaldehyde as the

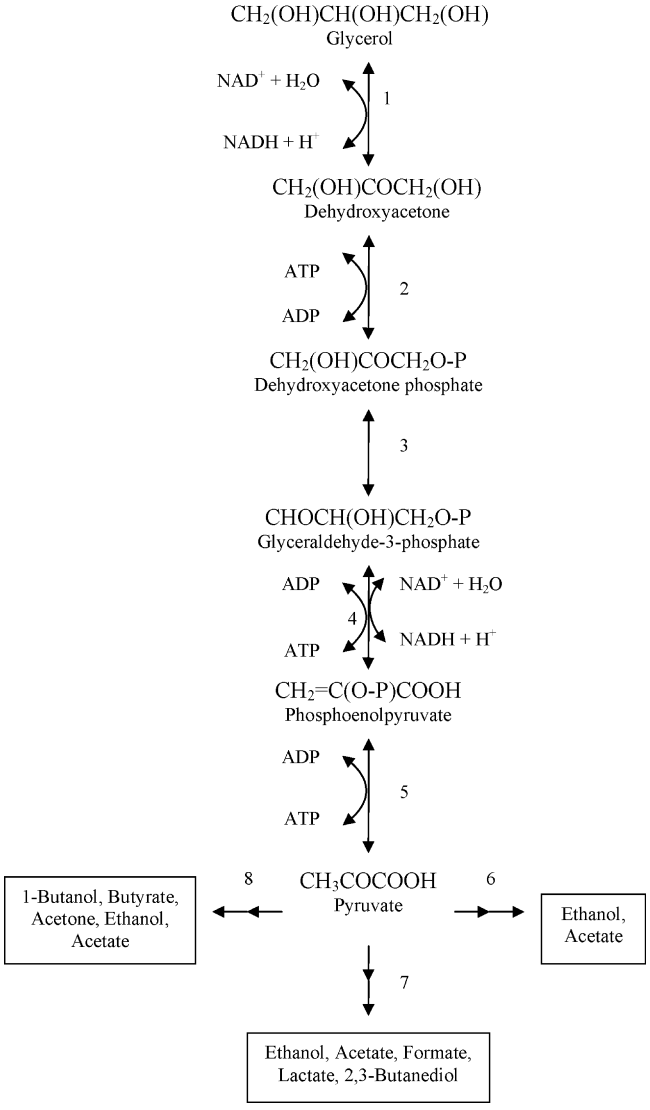


Fig. 7. Fermentation of glycerol with product formation as in carbohydrate fermentation as it proceeds for yeasts, enterobacteria and clostridia. 1, Glycerol dehydrogenase; 2, dehydroxyacetone kinase; 3, triose phosphate isomerase; 4, glyceraldehyde-3-phosphate dehydrogenase; 5, pyruvate kinase; 6, in yeasts ethanol is formed from pyruvate by the sequence of reactions as in Fig. 1; 7, in enterobacteria is proceeded the sequence of reactions as they are shown in Fig. 3; 8, in clostridia is proceeded the sequence of reactions as they are shown in Fig. 4.

intermediate (Fig. 1). Pyruvate can also be condensed to alpha-acetolactate and finally to acetoin and 2,3-butanediol (Fig. 2). In *C. butyricum* and clostridia strains, virtually two products are formed: acetate and butyrate. Small amounts of ethanol are also found. *C. pasteurianum* forms in addition 1-butanol which sometimes becomes the predominant product (Fig. 3) [101].

The second fermentation pathway for glycerol dissimilation results in the formation of propanediols [102,97]. It is presented schematically in Fig. 8. This pathway proceeds in two different branches followed by different bacteria species. The first branch (A) can be followed by a wide range of microbes of the *Enterobacteria* and *Clostridia* genera that are able to ferment glycerol to 1,3-propanediol under anaerobic conditions [97]. Initially, glycerol is dehydrated to 3-hydroxypropanal by glycerol dehydratase (a complex multi-subunit enzyme that requires coenzyme B12 as a cofactor) [103]. Then the 3-hydroxy propanal is reduced to 1,3-propanediol by an NADH linked 1,3-propanediol dehydrogenase.

The second fermentation branch (B) generates 1,2-propanediol (Fig. 8). This route has been studied mainly in *E. coli*, *C. splenoides*, *Thermoanaerobacterium thermosaccharolyticum* and *Thermoanaerobacter ethanolyticus* [104,105]. Glycerol is converted to l-dehydroxyacetone (acetol) and then to dehydroxyacetone phosphate, which is further dephosphorylated to methylglyoxal by methyl glyoxal synthase. The methylglyoxal could be reduced either to R-lactaldehyde as in *C. splenoides* or to hydroxyl acetone (acetol) as in *T. thermosacharolyticum* [106]. The lactaldehyde and acetol are then reduced to 1,2-propanediol [105,107].

The 1,2-propanediol formed is initially dehydrated to propanal by the enzyme diol-dehydratase. Propanal is then oxidized to propionyl-CoA by a CoA dependent aldehyde dehydrogenase. The next step is the transacetylation from propionyl-CoA to inorganic phosphate mediated by a phosphotransacetylase. The propionyl-phosphate is finally hydrolyzed to propionic acid by acetate kinase with concomitant phosphorylation of an ADP to ATP. This substrate-level phosphorylation is the only energy-generating step in the 1,2-diol fermentation. Meanwhile two equivalents of reducing power are produced. Thus, another molecule of propanal acts as hydrogen acceptor and yields 1-propanol. This backward reaction for the reoxidation of the reduced nucleotides has been supported also by other studies that have shown that propionate and 1-propanol are produced in equal molar amounts during this procedure [108].

### 6.1. Aspects of the glycerol assimilation pathways

The various pathways for microbial glycerol dissimilation that are schematically outlined in Figs. 7 and 8 are used simultaneously by the involved microorganisms, while ethanol and acetate are produced by all the implicated microbes [97]. Therefore, the product distributions are complicated functions of all the factors influencing cell physiology, gene regulation, gene product activities, etc. [97,101,102,104,105,107–109].

For instance, it has been shown in studies with *K. pneumoniae* cultures and glycerol as the carbon source that when glycerol is limiting large amounts of ethanol are produced, but as glycerol concentration increases ethanol formation ceases and the 1,3-propanediol approaches its maximum. In the same study, it has been shown that under acidic conditions the 2,3-butanediol fermentation is preceding while in pH uncontrolled cultures a product spectrum originating from all the possible reactions, can be obtained [100,109].

Another point that needs to be underlined is that microorganisms can either produce glycerol from glucose or convert glycerol to various products through the different

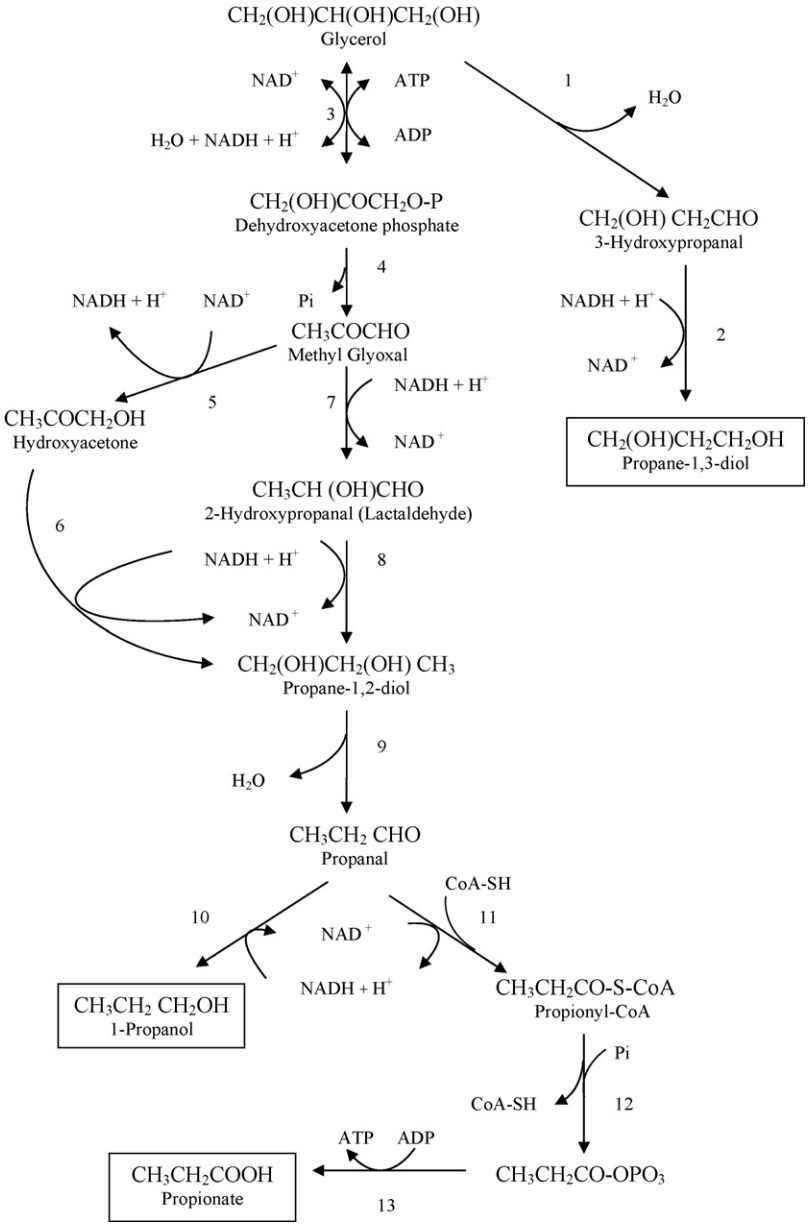


Fig. 8. Reductive fermentation of glycerol leading to the formation of propanediol, 1-propanol and propionate. 1, Glycerol dehydratase; 2, 1,3-propanediol dehydrogenase; 3, glycerol dehydrogenase/dehydroxyacetone kinase; 4, methyl glyoxal synthase; 5, methyl glyoxal reductase; 6, glycerol dehydrogenase; 7, glycerol dehydrogenase; 8, lactaldehyde reductase; 9, diol dehydratase; 10, alcohol dehydrogenase; 11, CoA-dependent aldehyde dehydrogenase; 12, phosphotransacetylase; 13, kinase.

pathways mentioned. Glucose to 1,3-propanediol conversion process has been achieved by a mixed culture process using yeast for glycerol production and Enterobacteriaceae for 1,3-propanediol production [110]. Therefore, a mixed and complicated culture of microbes as it is the putrefying corpse, could probably generate the products of propanediol fermentation (special attention to 1-propanol) with starting substrate glucose, part of which is initially fermented to glycerol. This possibility could explain partly the association of 1-propanol formation to glucose availability that was commended in the section of higher alcohols formation, as well as the fact that 1-propanol levels have been reported to be higher than the levels of the other higher alcohols.

## 7. Fatty acids as microbial substrates

A number of anaerobes such as Pseudomonades, Acinetobacter, Bacilli and Coliforms can grow on long-chain fatty acids [33,111,112]. They are usually degraded by β-oxidation [111,113]. The degradation of fatty acids through this procedure results in the formation of acetyl-CoA from even numbered fatty acids while odd-numbered fatty acids result to the formation of acetyl-CoA and propionyl-CoA [33]. The conversion of acetyl-CoA to acetone through the formation of acetoacetate by microbes has been already described in the Section 4.4 (Fig. 3). Moreover, in mammalian liver, acetyl-CoA can result in the formation of the so-called ketone-bodies

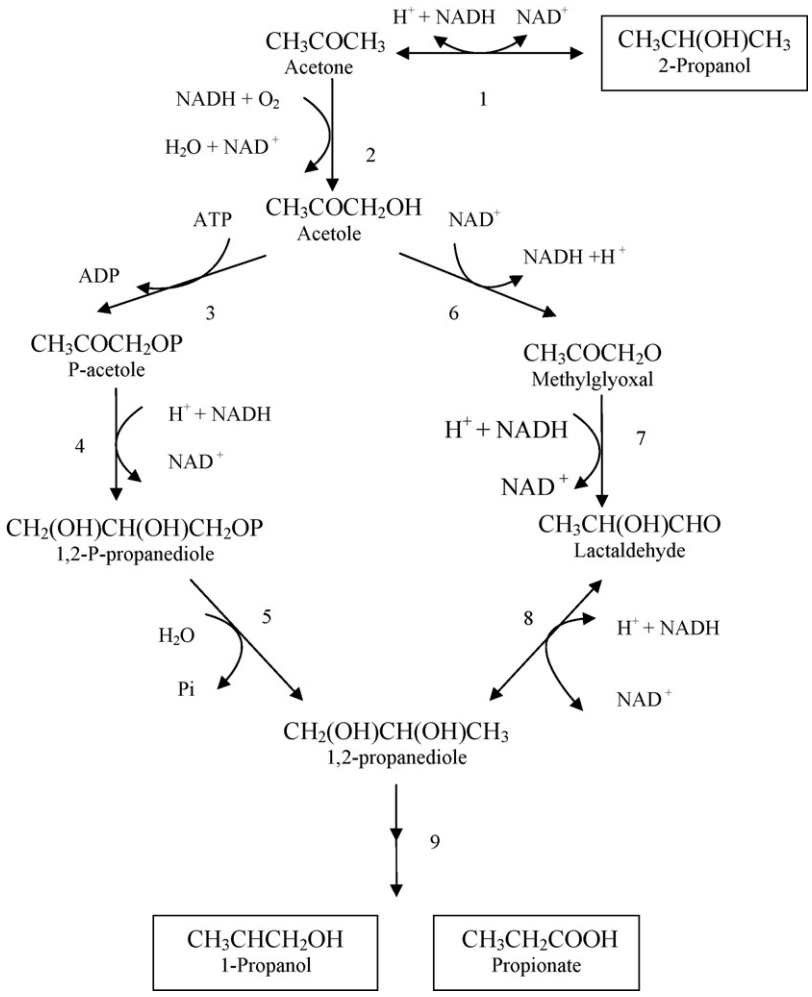


Fig. 9. Microbial acetone metabolism. 1, Alcohol dehydrogenase; 2, acetone monooxygenase; 3, acetol kinase; 4, 1,2-propanediol-1-phosphodehydrogenase; 5, 1-phospho-glycerol phosphatase; 6, acetole monooxygenase; 7, methylglyoxal reductase; 8, lactaldehyde reductase; 9, enzymes and reactions from 1,2-propanediol to the final products as in Fig. 8.

(acetoacetate, *d*-3-hydroxybutyrate and acetone) [114]. Elevated levels of ketone-bodies are found in circumstances such as fasting or diabetes or alcoholism [115].

Acetone, which is the most important and abundant ketone, from biological site of view, is a product as well as an intermediate metabolite of both bacterial and mammalian metabolic pathways [115–117]. Anaerobic bacteria of the *Clostridium* genus produce acetone as a major fermentation end product [116]. The metabolism of acetone by microbes can proceed in two different pathways as it is schematically outlined in Fig. 9.

In the first pathway, acetone could be reduced to isopropanol by secondary alcohol dehydrogenase in a $NAD^+$ dependent step [118]. In the second pathway, acetone can serve as a growth supporting substrate for a number of diverse aerobic [119] and anaerobic bacteria [120,121]. The second pathway involves two mechanisms for the acetone conversion: an oxygen dependent monoxygenase-catalyzed oxidation to produce acetol (hydroxyacetone) and/or a carbon dioxide-dependent carboxylase-catalyzed carboxylation to produce acetoacetate (the reverse reaction that forms acetone from acetoacetate) [121]. The mechanism of acetone conversion through acetol could result either to 1,2-propanediol with propionate and 1-propanol as the final products or to methyl glyoxal converted to lactaldehyde which gives further 1,2-propanediol [115].

The metabolism of propionyl-CoA can follow several different routes. It has been already referred its conversion to propionate (Fig. 8). *E. coli* and other bacteria can convert propionyl-CoA to pyruvate while most of them convert it to succinyl-CoA which enters in the tricarboxylic acid cycle [33].

## 8. Microbial production of other volatile compounds

Among the compounds that have been detected during the analysis of ethanol in post-mortem cases are included various ethyl esters which have been rather considered markers of ante-mortem consumption of ethanol [122,123].

Esters could be also formed by the microorganisms present during putrefaction. The microbial formation of esters can occur generally by the intracellular reaction between a fatty-acyl-coenzyme A and an alcohol catalyzed by an alcohol acyltransferase (or ester synthetase) [33]. The regulation and control of esters synthesis are not yet fully understood. It has been reported that the main factor controlling ester biosynthesis

is the expression level of the gene coding for alcohol acetyltransferase activity [124].

## 9. Discussion

This review on the microbial biochemical pathways generating volatile compounds co-detected during the forensic ethanol analysis supports the microbial formation of the following volatiles: ethanol, acetaldehyde, acetone, 2-propanol, 1-propanol, 1-butanol, isobutanol, isoamyl alcohol, *d*-amyl alcohol, acetic, propionic, butyric and isobutyric acids, and ethyl esters (mainly ethyl acetate). These volatiles result from the fermentation of the main substrates present in a putrefying corpse, namely carbohydrates, amino acids, glycerol and fatty acids. In Table 1 are presented the volatiles that could be produced from the various available substrates post-mortem, during the different fermentations proceeded, along with the microbe types that are able to carry them on.

Formation of volatiles from other substrates is not excluded, although we consider that the relevance to their final concentration and to the ethanol analysis is much less important. The microbial production of volatiles from ethanol as substrate has not been considered in this report (reviewed in [66]). However, we are of opinion that ethanol assimilation from microbes is of importance in the advanced stages of putrefaction, after the preferred main substrates have been exhausted, since ethanol consumption by bacteria, in the presence of other substrates, is thermodynamically unfavorable [67].

The detection of volatile compounds has been reported in corpses from aircraft accidents [13,125,126], recovered from water [127,128] or routinely autopsied [10,11,14,129]. Moreover, various volatiles have been detected in vitro studies with post-mortem specimens or in studies with putrefying animals [5,18,130,131]. In cases concerning chronic alcoholics and diabetics whose death was attributed to alcoholic ketoacidosis has been also reported the presence of various volatiles [132,133]. Furthermore, volatiles have been detected in the blood of living subjects either after the consumption of alcoholic beverages [134–137] or as products of metabolic processes in diabetic patients or healthy controls [115,134,137–140]. In the blood of drunk drivers which had BAC $> 0.46$ g/L were found ethyl acetate, methanol, 2-propanol, acetone, 2-butanol, butanone and 1-propanol [137]. It has been also reported the presence of ethanol, 1-propanol, isobutanol, butanol and *d*-amyl-alcohol in urine from diabetic patients as well as healthy controls [138].

Most previous studies concerning post-mortem volatiles in general, list only which volatiles were screened for and not which were actually found nor their concentrations [5,6,17–19,125–130]. It has been reported the detection of methanol, formaldehyde, 1-propanol, 2-propanol, acetone, propionate, acetate, acetaldehyde, butyrate and isobutyrate, isobutanol, 1-butanol, 2-butanol, isoamyl alcohol and *d*-amyl-alcohol concomitantly with ethanol. On the other hand, it has been reported the post-mortem presence of formaldehyde, acetone and acetaldehyde in the absence of ethanol [5,18].

The detection of the volatile acids acetate, propionate, butyrate and isobutyrate has been reported in only three relevant studies [1,5,18]. This fact should be attributed to their delayed elution from the column during volatile chromatographic analysis which has as consequence that the post-mortem specimens are not routinely screened for their presence. The post-mortem detection of branched-chain-alcohols has been reported in only one study [130] while they have been also detected in living subjects [138]. The presence of esters in post-mortem specimens is considered post-mortem marker of ante-mortem ethanol consumption [122,123] and, as a result post-mortem studies do not correlate them with microbial production.

The microbial production of methanol (and formaldehyde) is not supported by the provided biochemical data although it has been detected in post-mortem cases [5] and in living subjects [137,140]. The presence of 2-butanol and butanone in post-mortem cases (reported in [125,137]) should also not considered a result of microbial activity. Butanone can be

Table 1
Fermentative volatile products generating during the respective fermentations of the post-mortem substrates and the types of microorganisms able to carry them on

| Substrate/fermentation | Types of microbes | Products |
|---|---|---|
| Carbohydrate | | |
| - Ethanol | Yeasts, bacteria | Ethanol, acetaldehyde, acetate, esters |
| - Lactate | Lactobacilli, enterobacteria, enterococci, streptococci, bifidobacteria | Ethanol, acetaldehyde, acetate, propionate, 1-propanol, esters |
| - Mixed acid, butanediol | Enterobacteria | Ethanol, acetaldehyde, acetate, and little propionate, 1-propanol, esters |
| - Butyrate, butanol, acetone | Fusobacteria, propionobacteria, clostridia | Acetone, 2-propanol, 1-propanol, 1-butanol, propionate, butyrate, esters |
| Amino acids | Yeasts, enterococci, clostridia | 1-Propanol, *d*-amyl-alcohol, isoamyl alcohol, isobutanol, ethanol, esters, acetaldehyde, propionate, isobutyrate |
| Glycerol/propanediol | Yeasts, lactobacilli, enterococci, enterobacteria, clostridia | Ethanol, acetaldehyde, 1-butanol, acetone, 2-propanol, 1-propanol, acetate, propionate, butyrate, esters |
| Fatty acids | Yeasts, enterobacteria, clostridia | Acetone, 2-propanol, 1-propanol, propionate, ethanol, acetaldehyde, acetate, esters |

produced by microbes from 2-butanol and vice versa only if one of these compounds is present as substrate in the medium [118]. The presence of methanol, 2-butanol and butanone should be considered rather the result of either ante-mortem exposure (consumption of alcoholic beverages or other) or contamination from an unusual source as reported by Caughlin [141]. We are in agreement with the consideration expressed by O'Neal and Poklis [1] that ''it appears that any organic volatile may be present in post-mortem specimens as the result of exogenous contamination''. A recent study reporting the presence of over 80 volatile substances in the tissues of two post-mortem cases supports further this consideration [142]. All the volatiles that we support that could be produced by microbes are included in the detected substances along with many others, though of unspecified origin—ante-mortem exposure, post-mortem formation or post-mortem contamination.

### 9.1. Internal standard selection

Nowadays ethanol analysis is performed routinely by head space gas chromatography and detection in most cases with FID detector. Between the compounds that have been used as internal standards in ethanol analysis in forensic laboratories are included 1-propanol, *t*-butanol, butanone, isobutanol and acetonitrile [1,143–145]. However, later on, the use of 1-propanol as internal standard has been considered insufficient since it is a major putrefactive product that should be rather considered as a marker of post-mortem ethanol synthesis [1,143,146]. The data presented in this review make obvious that both 1-propanol and isobutanol could be produced from microbes, the first in more significant amounts than the latter. For this reason, we suggest that both these compounds should not be used as internal standard.

The use of butanone or *t*-butanol has been considered a safer choice for internal standard compared to 1-propanol [143]. Butanone however, has been found in post-mortem cases [141]. As regards *t*-butanol, its presence in post-mortem biological specimens is still controversial [13,125,126]. The microbial production of *t*-butanol and butanone is not supported by our review and consequently both can be considered proper internal standards considering that there is no case of external contamination of the analyzed specimen. In our opinion, the use of acetonitrile is the most appropriate choice for internal standard in ethanol analysis since it is by no means a product of microbial activity. Moreover, its detection in post-mortem cases has not been reported yet nor it is an ingredient of any alcoholic beverages. Furthermore, it fulfils all the criteria that should an internal standard have for the ethanol analysis. In the authors' laboratory acetonitrile is used as the internal standard during forensic ethanol analysis in a well validated procedure.

### 9.2. Interpretation of ethanol analysis results

In the forensic literature it is reported the detection of only few volatiles compared with the spectrum of volatiles that could be formed [5,6,17–19,125,130,137–138]. In most of the relevant studies, besides ethanol, have been detected 1-propanol, acetaldehyde, acetone, 2-propanol, isobutanol and 1-butanol. It looks likely that these volatiles predominate between all the possible volatiles that could be formed post-mortem and actually are formed. It is worth mentioning that the presence of volatiles in post-mortem specimen has been included among the criteria that could specify the origin of post-mortem ethanol [1,20]. More specifically, they have been considered as biochemical markers in order for a post-mortem specimen to be flagged as suspicious for microbial contamination, if present in abnormally high concentrations [19]. Unfortunately, these ''abnormally high concentrations'' are not specified.

Regarding the concentration of the post-mortem formed volatiles there are not reported values except for ethanol and 1-propanol. The concentration of the post-mortem formed ethanol it is more possible to be lower than 0.7 g/L and usually less than 0.3 g/L [1,26]. The concentration of 1-propanol in post-mortem blood as a result of microbial production has been reported to range from 0.03 to 0.07 mg/L [1]. This volatile has been considered the most correlated to microbial post-mortem ethanol formation [17,19,127,147]. The ratio of ethanol to 1-propanol concentration has been used to verify the post-mortem production of ethanol and the quantitative pattern between the produced ethanol and 1-propanol [127,147,148]. This strategy however has been questioned by a recent study where ethanol was produced from glucose by *C. albicans* in post-mortem blood [149]. Another volatile whose presence in bodies recovered from water has been considered a good indicator of post-mortem ethanol formation is 1-butanol [128], a compound that could actually be a product of post-mortem microbial activity.

As could be concluded from Table 1, the fermentations that result to ethanol formation (namely ethanol, lactate, butanediol and mixed acid fermentations) are carried out by microorganisms which expand and grow from the early stages of putrefaction (yeasts, etc.). This is in accordance with previous studies reporting that ethanol production could occur even in hours after death, if the conditions are favorable, and could continue for some days before a decline in ethanol concentration is observed [1,2,6]. The presence of other volatile compounds in the corpse in the starting point of microbial ethanol production should be possibly attributed either to exogenous sources, e.g. ante-mortem consumption for ethanol, acetaldehyde, 1-propanol, 2-propanol, methanol, branched-chain alcohols or due to endogenous sources, e.g. diabetic or fasting subjects, for acetone, 2-propanol.

However, as the putrefaction proceeds and the anaerobic micro flora expand the action of clostridia species predominates in the corpse. Simultaneously, carbohydrates which are the preferred substrate for microbes are exhausted and amino acids, glycerol and fatty acids remain to be consumed by the microbes. As a consequence, ethanol production decreases or ceases, its levels reach a maximum and the production of 1-propanol, 2-propanol, acetone, 1-butanol and branched-chain alcohols increase, increasing their concentration too.

All the relevant studies concerning post-mortem volatiles demonstrate that in cases involving decomposing bodies could

not be present or seldom present in detectable quantities all the possible volatiles. The various formation rate reported for post-mortem volatiles could be well supported by the data presented in this contribution. Corpses in different stages of putrefaction, depending on difference in the environmental conditions, are attacked by different microbial species and their action results in the formation of variable and unpredictable volatile levels.

At this point is needed to be underlined the fact that all the volatiles that could be microbial products are minor ingredients of alcoholic beverages [47,69,83,136,150]. Consequently, the ante-mortem consumption of such beverages would probably result in detectable amounts of these volatiles along with considerable ethanol amounts after death.

The data we provide support that ethanol can be produced from all the available substrates in the post-mortem, however, with higher rates and yields from carbohydrates and during the early stages of putrefaction. The production of 1-propanol could result also from all the available substrates, however, with greater yields from the fermentations of amino acids, glycerol and fatty acids and could last even till the advanced stages of putrefaction when the clostridia predominate (Table 1). Therefore, 1-propanol could still increase when ethanol has reached its maximum or even it starts to decline. This might be the reason why 1-propanol is produced in larger quantities than the other volatiles and it has been detected in all post-mortem cases when ethanol production has been considered.

## 10. Concluding remarks

The significance of the presence of volatiles in post-mortem cases for the interpretation of the ethanol analysis results is up to date controversial. Most of the relevant studies report only qualitative determination of only few of the possibly produced compounds, due to either the low concentrations they present in decomposing bodies or the fact that the applied methods could not routinely detect them.

So far, the information regarding the concentrations of the volatiles that have been demonstrated in this review that could be produced in post-mortem cases is missing. Such data might be valuable in assisting the interpretation of ethanol results in cases with putrefaction. In our opinion, the performance of studies in order to establish the minimum volatile levels which could indicate microbial contamination and subsequent ethanol production, as well as the correlation of these levels with the ethanol concentration produced and possibly the stage of putrefaction could be potentially helpful in this direction.

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