Eubiotics for Food Security at Farm Level: Yeast Cell Wall Products and Their Antimicrobial Potential Against Pathogenic Bacteria

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Abstract

The population increase in the last century was the first cause of the industrialization of animal productions, together with the necessity to satisfy the high food demand and the lack of space and land for the husbandry practices. As a consequence, the farmers moved from extensive to intensive agricultural systems and introduced new practices, such as the administration of antimicrobial drugs. Antibiotics were then used as growth promoters and for disease prevention. The uncontrolled and continuous use of antibiotics contributed to the spread of antibiotic resistance in animals, and this had adverse impacts on human health. This emergence led the European Union, in 2003, to ban the marketing and use of antibiotics as growth promoters, and for prophylaxis purposes from January 2006. This ban caused problems in farms, due to the decrease in animal performances (weight gain, feed conversion ratio, reproduction, etc.), and the rise in the incidence of certain diseases, such as those induced by *Clostridium perfringens*, *Salmonella*, *Escherichia coli*, and *Listeria monocytogenes*. The economic losses due to the ban increased the interest in researching alternative strategies for the prophylaxis of infectious diseases and for health and growth promotion, such as feed additives. Yeast-based materials, such as cell wall extract, represent promising alternatives to antibiotics, on the base of their prebiotic activity and their claimed capacity to bind enteropathogenic bacteria. Several authors reported examples of the effectiveness of yeast cell wall products in adsorbing bacteria, but there is a lack of knowledge on the mechanisms involved in this interaction. The purpose of this review is to provide an overview of the current approaches used for the control of pathogenic bacteria in feed, with a particular focus on the use of yeast-derived materials proposed to control zoonoses at farm level, and on their effect on animal health.

Keywords: biosecurity, antibiotics, yeast cell wall, growth promoters, eubiotics, feed

Biosecurity from Farm to Fork

THE DEVELOPMENT OF FARMING POLICIES and interventions for sustainable human food production has the aim to ensure a healthy food source for animals and people. Sustainability is, therefore, a topic of major concern in the biosecurity of food productions from farm to table. Reducing public health risks from health issues like zoonoses at the human-animal ecosystem interface (such as antimicrobial resistance) is crucial in preventing new, emerging, and re-emerging hazards (Saegerman *et al.*, 2012).

Infections caused by pathogens that contaminate the food supply are an example of the complex web that links humans with animals, plants, and microbial populations all around the world (Behravesh *et al.*, 2012). The microbial pathogens that can contaminate food are mostly maintained in human and animal reservoirs and contaminate the food supply through the excreta of infected humans and contaminated food. Example of

reservoirs for pathogenic microorganisms in the food products are meat, milk, or eggs. Xenobiotics invade the food chain because they are present in the excreta or in the carcasses of infected animals (Alum *et al.*, 2016). Furthermore, some pathogens have the capacity to persist in the environment (water, soils, etc.), or in multiple hosts, and can contaminate food through pathways that reflect the variety of ecosystems that characterize the food supply chain. Safety in the food chain depends on studying and thus understanding these complex pathways well enough to prevent the risks (WHO, 2015).

Some diseases that infect animals can be passed to humans (zoonotic diseases), and animal-derived food is the most common vehicle of infections, mainly because the infected animals often appear healthy on inspection at slaughter (Greger, 2007; Bidaisee and Macpherson, 2014). High attention in food security during the 20th century and efforts in animal disease control have significantly reduced the incidence of infections related to foodborne diseases (EFSA, 2015). Addressing these

pathogenic microbes requires effective prevention strategies, mainly based on reducing the levels of microbial contamination throughout the food chain, and on maintaining biosecurity in the farm or ranch where animals are raised (Behravesh *et al.*, 2012). Livestock health maintenance is, therefore, an economic issue not only for the breeders but also an important key factor in the biosecurity of the food chain from farm to fork.

Antibiotic Use in Livestock

The discovery of the positive effects of antibiotics on farm animals' health (Funtaine and Atkeson, 1950; Stokstad and Jukes, 1950; Carpenter, 1951) coincided with the globalization and industrialization era, with the rapid growth of the population after 1950 that faced an increase in food production to fulfill the increasing nutritional demands. Farming activities changed from an extensive to an intensive form, to fulfill the increasing demand for food and the decreasing availability of agricultural lands (Huyghebaert, 2005; Godfray and Garnett, 2014; Landert *et al.*, 2017). In addition, the trend to minimize losses and outgoings, with the aim to maximize the earnings, led to focus on the improvement of animal performances. In this sense, the management of animal production evolved, and modern farms started to use new systems to push the growth rate of animals to their maximum, and increase disease prevention since the confined breeding enhanced the transmission of infectious agents (Perry *et al.*, 2013). As a consequence, the use of antimicrobial growth promoters (AGPs) for metaphylaxis and/or treatment of the most common animal diseases started to diffuse among farmers, together with new systems such as improved husbandry, genetics, and nutrition practices.

AGPs are proposed to exert their action in favor of animal performances in different ways: by reducing the incidence and severity of subclinical infections; by reducing the microbial use of nutrients; by altering gut motility to enhance a better assimilation of nutrients, and to improve their absorption; by increasing the growth rate and nutrient assimilation efficiency by reducing the amount of growth-depressing metabolites produced by Gram-positive bacteria (Gaskins *et al.*, 2002; Dibner and Richards, 2005; Castanon, 2007; Niewold, 2007).

The Spread of Antimicrobial Resistances

When the first evidences on the positive effects of antibiotic drugs on swine, poultry, and cattle aroused (Funtaine and Atkeson, 1950; Stokstad and Jukes, 1950; Carpenter, 1951), the use of antibiotics to mass treat a large number of animals became practical. This tendency derived and was supported by meat producers that were anxious to protect their investments. Food and Drug Administration in USA (US FDA) approved these drugs as growth promoters for animals in 1951 (Hao *et al.*, 2014). Furthermore, antibiotics are more economical and effective when used as early as possible, rather than when fully developed disease is evident (Gustafson and Bowen, 1997). The necessity to improve animal performances and prevent some gastrointestinal diseases caused by pathogens such as *Escherichia coli*, *Salmonella*, and *Clostridium* (Satterthwaite *et al.*, 2010; Laxminarayan *et al.*, 2013) led to massive uncontrolled use of antibiotics in livestock as AGPs and for prophylactic purposes. As a consequence of a high degree of selective pressure on pathogenic and commensal bacteria (Ghosh and LaPara, 2007), ideal conditions for the emergence and spread of antimicrobial resistant bacteria in

animals were created (Aminov and Mackie, 2007). The direct consequence was the ineffectiveness of AGPs usage for the treatment of infectious diseases (Aminov and Mackie, 2007; Ghosh and LaPara, 2007; Courvalin, 2008; Wegener, 2012; Ventola, 2015). The spread, in farm animals, of antimicrobial resistances toward those pathogens had consequences also in human health, since the resistances started to be transmitted to humans by the food chain (Boyce, 2008; Rosenblatt-Farrell, 2009; Marshall and Levy, 2011; Ventola, 2015).

In 2003, the European Parliament took action against the overuse of antibiotic drugs in livestock, by the redaction of the Regulation 1831/2003 on additives for use in animal nutrition. The Regulation 1831/2003 stated that antibiotics could be marketed and used as feed additives only until December 31, 2005 (EU 2003). After this date, the usage of these substances as growth promoters and for prophylaxis purposes was banned. The usage of medical substances in animal feeds would have been limited to a therapeutic use by veterinary prescription (Ungemach *et al.*, 2006). The restraint of the antimicrobial resistance emergence took to the settlement of another problem: the incidence of gastrointestinal animal diseases, caused by microbial pathogens, increased in countries where the usage of AGPs was stopped (Singer *et al.*, 2003).

Natural Products as Alternatives to Antibiotic Growth Promoters

The position taken by the European Union immediately created the necessity for innovative strategies and products as alternatives to AGPs, to fulfill the gap left by the ban of antibiotics. For this purpose, the interest in the use of natural compounds as feed supplements increased, since their claimed prebiotic and antimicrobial features can be exploited by scientists to ensure the wellness of animals, and consequently to improve performance and maximize production (Jouany and Morgavi, 2007; Windisch *et al.*, 2008).

Substitutes to antibiotics are of primary importance in the field of animal nutrition, veterinary medicine, and feed industry (Thornton, 2010). Feeds containing no chemical additives are increasingly used in farm animal's nutrition, and a number of natural substances are being used or researched for their claimed antimicrobial effect in feed. These substances act at the level of the intestine, binding (or somehow inhibiting) pathogenic bacteria, viruses, and toxins, which are eliminated by the feces, while preserving the beneficial intestinal flora (Huyghebaert, 2005). In addition, many of these substances also have antioxidant and immune-stimulant properties, offering protection against the colonization of the intestine by bacterial pathogens like *Salmonella*, *Listeria monocytogenes*, *E. coli* and their toxins (Zhang *et al.*, 1992; Murugesan *et al.*, 2015; Yang *et al.*, 2015).

Alternatives to growth promoters should have the same beneficial effect as the AGPs, although it is not always clear how these products exert their beneficial action. Numerous eubiotics proposed as AGPs are available on the market, and while some products clearly have antimicrobial potential, the mechanisms underlying their efficacy are not yet known (Thacker *et al.*, 2013). Several alternatives for AGPs with different mode of action currently exist (Huyghebaert *et al.*, 2011): (1) *Exogenous enzymes* (Ravindran, 2013); (2) *Organic acids* (Cherrington *et al.*, 1991); (3) *Probiotics or "direct-fed microbials"* (Chaucheyras-Durand and Durand,

2009); (4) *Phytogenic feed additives* (Yang *et al.*, 2015); (5) *Clay minerals* (Williams and Haydel, 2010); (6) *Prebiotics*, like inulin and fructooligosaccharides, which can have a beneficial action with selective stimulation of the growth or metabolic activity effects on some species of the intestinal microbiota (Sadeghi *et al.*, 2013; Caipang and Lazado, 2015; Fowler *et al.*, 2015). Among prebiotics, the oligo-saccharides of yeast cell wall (YCW) origin have been proposed as replacement for AGPs, for their capacity to stimulate animal performances because of their immuno-modulatory and antimicrobial effects (Ghosh *et al.*, 2012; Ganner *et al.*, 2013).

YCW Products as Alternative to Antibiotic Growth Promoters

Yeasts are probably one of the earliest domesticated organisms. People have used yeasts for fermentation and baking throughout history. Yeast extract has importance as a flavor enhancer due to its high content of glutamic acid (Populin *et al.*, 2007). Yeast powder or yeast drinks are proposed as health-improving food supplements because of their high content in vitamins, proteins, and minerals (Ghosh *et al.*, 2012). Yeast products have been fed to animals for more than hundreds years and include yeast-fermented mash directly produced on the farm, yeast by-products from breweries and distilleries, or yeast products commercially produced for animal feeding. After EU ban of AGPs, and as a consequence of scientific studies on the efficacy of YCWs as growth promoters, the market of yeast derivatives grew. The global market for yeast ingredients has been estimated to 1.7 billion of USD in 2014 and is projected to grow at a CAGR (Compound Annual Growth Rate) of 8.2% from 2015 to 2020. In 2014, the global consumption of yeast ingredients was 213.8 kton and is projected to grow at a competitive CAGR of 8.0% during the forecast period (Market-sandMarkets, 2015). The European region accounted for the largest share in the market for yeast ingredients in 2014, owing to the heavy demands for natural ingredients from the flour-ishing processed food market of the region.

Mechanisms of Action of YCW Products Against Target Pathogens

The effect of YCW-derived products, such as mannan-oligosaccharides (MOS), has been studied in several *in vivo* studies, in which their prebiotic effect on animal perfor-mances was evaluated in the presence of viral or bacterial infections. Mannoproteins and their branched carbohydrate portion are responsible for pathogen-host recognition and for the interactions with the environment, and determine the immunological specificity of the yeast. The majority of the mannoproteins are covalently linked to the inner glucan layer (Zlotnik *et al.*, 1984; Lipke and Ovalle, 1998). The compo-sition of *Saccharomyces cerevisiae* cell wall is represented in the scheme in Figure 1.

Several studies have demonstrated the effect of live yeast cells and of products derived from the YCW, in improving the innate immune response and in reducing the incidence of infectious disease in farm animals, thus justifying their use as an effective replacement strategy for the prevention of
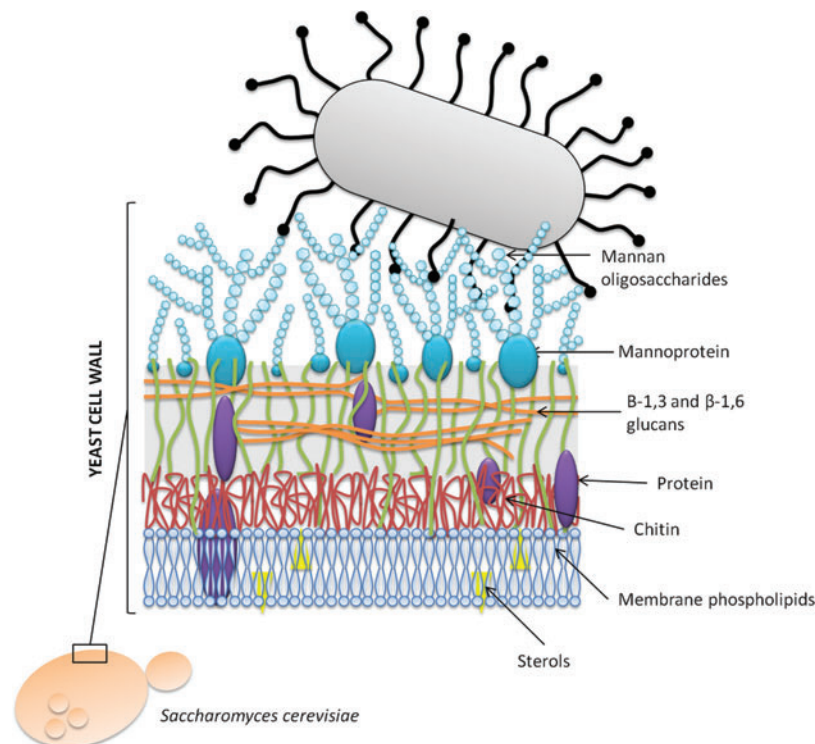

FIG. 1. YCW structure and antiadherence strategies by YCW-derived products, and simplified relationships among the components of *Saccharomyces cerevisiae* cell walls and adhering bacteria. The specific lectins of pathogens for mannose residues are the type I fimbriae. As adhesion is the reason for bacterial colonization in the gastrointestinal tract, it can be used for removal of pathogens by agglutination. The mannan-oligosaccharides of YCW are proposed to bind the fimbriae of the pathogens and inhibit their multiplication. YCW, yeast cell wall. Color images available online at www.liebertpub.com/fpd

infectious disease in farm animals, with effects identical to previously used AGPs (Perić *et al.*, 2009; Haldar *et al.*, 2011; Ghosh *et al.*, 2012). The dietary supplementation with YCWs has been proven to stimulate the systemic innate immune responses of broiler chickens (Sadeghi *et al.*, 2013), suggesting the role of these products in regulating immune homeostasis (Alizadeh *et al.*, 2016).

MOS obtained from the cell wall of *S. cerevisiae* act also as high-affinity ligands for microorganisms (Spring *et al.*, 2000). The inhibitory effect of YCW products versus pathogenic bacterium adhesion to the mucosal surface of the intestine is hypothetically due to the ability of certain bacteria, such as *E. coli*, *Salmonella* Typhimurium, and *Salmonella* Typhi, with mannose-binding fimbriae to bind mannoproteins within YCW (Tiago *et al.*, 2012). The branched lateral chains of the MOS are organized in a three-dimensional structure that represents the biologically active conformation of the MOS. This structure provides alternative sites for the adhesion of pathogens (Ofek *et al.*, 1977). Different studies showed that pathogenic microbes having mannose-specific fimbriae can bind through type-1-fimbriae to mannose, although this mechanism is not fully understood (Shoaf-Sweeney and Hutkins, 2008).

According to the current proposed mechanism for YCW interference with bacterial infections, the ingestion of YCW products might supply with competitive attachment sites (such as MOS) for the host receptors, thus reducing the risk of the pathogenic bacteria to colonize the intestinal tract and preventing infection (Costerton *et al.*, 1978; Lipke and Ovalle, 1998; Sadamoto *et al.*, 2004). Bacteria bound to MOS in the intestinal tract can pass through the gut, instead of attaching to host epithelial cells (Caipang and Lazado, 2015). The hypothesized mechanism of the pathogen cell adhesion to the mannan chains is represented in Figure 1.

In Vitro Evidences of the Efficacy of YCW Products

The interaction between YCW and bacteria has been studied *in vitro* and *in vivo*. The various nature of these products, which can differ from the originating yeast species and strain, makes the *in vitro* testing a useful screening method before *in vivo* studies, although it is still difficult to define a standard procedure for the evaluation of YCWs efficacy (Ganner *et al.*, 2013).

Pérez-Sotelo *et al.* (2005) reported that the *S. cerevisiae* strain Sc47 binds *in vitro* to strains of *Salmonella* that express type I fimbriae. By performing an agglutination test, the authors visually observed an evident sedimentation of the bacterium-YCW complexes, as well as an adhesion of *Salmonella* cells to the yeast cells by transmission electron microscopy. A total of 57.7% of the isolates and of the *Salmonella* serovars tested adhered to the product used in their study. These observations suggest that the adhesion of *Salmonella* onto the surface of *Saccharomyces* cells could, in part, explain the protective effect of some yeast probiotics.

A quantitative method to monitor bacterium adhesion to yeast-derived products was developed by Ganner *et al.* (2013). The method is based on the immobilization of the tested YCW product in the well of a microplate, measuring the turbidity of culture solutions in the same wells, using the optical density as growth parameter of adhering bacteria. Subsequently, the optical density was related to colony-forming unit (CFU) count by linear regression. The authors

tested the YCW fractions from a strain of *Trichosporon mycotoxinivorans* for its ability to bind Gram-negative pathogens such as *Salmonella*, *E. coli*, and *Campylobacter* strains and Gram-positive probiotic bacteria of the genera lactobacilli and bifidobacteria, as well as the Gram-positive pathogen *Clostridium perfringens*. The study reported that 7/10 *Salmonella* Typhimurium and *Salmonella* Enteritidis strains that were tested adhered to the cell wall product with an amount between 10^3 and 10^4 CFU/10 μg. Four out of seven *E. coli* strains showed a lower average binding capability (10^2 CFU/10 μg), whereas *Campylobacter jejuni* and *C. perfringens*, as well the bacteria of the genera lactobacilli and bifidobacteria did not bind to the YCW, thus revealing and confirming that YCW has a selective effect against some pathogenic bacteria, while having no adverse effect on beneficial and commensal bacteria. These data are supported by *in vivo* evidences of MOS-induced increase in broiler cecal populations of lactobacilli and bifidobacteria, as well as in the genus *Faecalibacterium*, in support of the evidence of the positive effects of MOS on the beneficial bacteria (Baurhoo *et al.*, 2007; Park *et al.*, 2016).

Tiago *et al.* (2012) showed that the phenomenon of yeast-bacterium adhesion occurred both *in vitro* and *in vivo*. The authors used germ-free mice as animal model to confirm and visualize the interaction between the yeast and pathogenic strains of *E. coli* and *S. typhimurium* on the intestinal epithelium. By using a gnotobiotic animal, they observed the inter-relationships occurring between yeast and the target microbial strains in the gastrointestinal ecosystem, without the interference of the complex autochthonous microbial flora. The images obtained by scanning electron microscopy showed the adhesion between the enteropathogenic bacteria and the yeast on the intestinal epithelium. The authors also proposed that a chemotaxis phenomenon could be involved in the interactions between the bacteria and yeasts since the bacteria seemed to be attracted to the yeast surface in the presence of yeast.

Bacterial adhesion can also occur without fimbriae, namely by afimbrial adhesins that are not organized in fimbriae, but instead seem to be attached directly to the bacterial surface as single proteins or large multiunits aggregates (Krogfelt, 1991; Klemm, 1994), but little more is known about the mechanisms of these adhesive interactions through lectins or protein–protein binding. More informations about the composition of fimbriae, adhesins, and about the characteristic structure of the MOS are necessary to better understand the biochemical interactions with the MOS (Krogfelt, 1991). Furthermore, other complex factors, such as the YCW components, the strain characteristics, and the nonspecific interactions, must be taken into account, to find the appropriate strategies and methods to be applied for *in vitro* testing, to enhance the binding between the YCW derivatives and pathogenic bacteria and elucidate all the characteristics of the antimicrobial effect of these compounds on bacterial pathogens (Klemm, 1994; Sadamoto *et al.*, 2004; Trevisi *et al.*, 2012). These findings could also guide the *in vitro* selection of the yeast strains to be used as alternative to AGPs, to address their antimicrobial activity toward several pathogens.

In Vivo Evidences of the Efficacy of YCW Products

The effect of yeast live cells and derived products in improving the innate immune response was demonstrated *in vivo* (Perić *et al.*, 2009; Haldar *et al.*, 2012; Ghosh, 2013). Under a *Salmonella enteritis* challenge, Sadeghi *et al.* (2013) demonstrated that

YCW stimulates the systemic innate immune responses of broiler chickens. Improvement in host immune response was also found with dietary supplementation with MOS of nursery pigs experimentally infected with porcine reproductive and respiratory syndrome virus (Che *et al.*, 2011). The dietary supplementation of MOS in broilers diet has been proven to inhibit the replication and shedding of the avian influenza virus H_9N_2, thus reducing its morbidity, due to a positive effect on the immune response of the host (Akhtar *et al.*, 2016). Hence the use of MOS may constitute a novel and useful alternative to reduce environmental contamination and spread of viruses.

In a recent study, Fowler *et al.* (2015) evaluated the growth-promoting effects of prebiotic YCW products in starter broilers under an immune stress and *C. perfringens* challenge. A significant improvement in growth rate, increased body weight, and improved feed conversion was observed, compared to control birds that were not fed with YCW, with an optimum dose of ~ 250 ppm of YCW product.

Similar results were also obtained in recent studies (M'Sadeq *et al.*, 2015; Xue *et al.*, 2017), where the researchers tested the effect of YCW products in reducing the incidence of the necrotic enteritis induced by *C. perfringens* in poultry. In their experiments, they showed that YCW products were all effective in mitigating performance decline, mortality, and lesions associated with the necrotic enteritis. Furthermore, they demonstrated that YCW suppressed inflammatory response, promoted generation of immunoglobulin, and increased the production of short-chain fatty acids, thus suggesting potential benefits to bird health. These results put in evidence the differences in the mode of action of YCW derivatives compared to the AGPs. Antibiotics have a direct effect on the organisms and are directly bactericidal, causing death of sensitive bacteria, or bacteriostatic, preventing bacterial growth. YCW products act at different levels by enhancing immunity and exerting a positive effect on the gut microflora, with a consequent reduction of the damages induced by clostridia. It is therefore essential to underline that the aim of using alternatives such as YCW is to maintain performance and health, while preventing or minimizing mortality and morbidity when there is a *necrotic enteritis* challenge in an antibiotic-free production situation (Thanissery *et al.*, 2010; Xue *et al.*, 2017).

In another study, Andrés-Barranco *et al.* (2015) demonstrated that feed supplementation with YCW products may be a useful complementary tool for the control of salmonellosis in fattening pigs, and observed that the addition of a dosage higher than 2 kg/t of the YCW product to the pig diet during the entire fattening period was associated with a reduction in *Salmonella* prevalence, shedding, and seroconversion.

Adding MOS from the cell walls of baker's yeast to the diet of Atlantic salmon fed with extracted soybean meal and/or extracted sunflower meal, Refstie *et al.* (2010) observed 10% better feed efficiency ratio (the ratio between the weight gain and the consumption of dry matter from the feed), 8% faster growth, and 11% higher protein retention. Dietary soybean meals are known to alter the number and diversity of intestinal bacteria in salmonid fish, thus inducing the growth of unfavorable bacteria that worsen the inflammation. In the same study, the authors demonstrated that the YCW product supplementation in the diet of salmons induced the reduction of diarrhea, and most noteworthy, the elimination of the soybean meal-induced enteritis, clearly demonstrating a positive effect of YCW on gut health of Atlantic salmon.

Conclusions

Antibiotics have been widely used in animal production for decades. Some were used therapeutically for the treatment of specific animal diseases; most were given for prophylactic purposes and as AGPs, to improve growth rate, performance, and feed conversion efficiency. However, due to the emergence of microbe resistance to antibiotics that are used to treat human and animal infections, the European Union banned, from January 1, 2006, the marketing and use of antibiotics as growth promoters in farm animals. The ban together with a growing interest by the consumers, all over the world, in healthy and antibiotic-free food, and the considerable economic losses in livestock farming, due to animal with gastrointestinal diseases, led to greater interest in alternatives to AGPs. Promising alternative substances to AGPs for the control of pathogenic bacteria are YCW-based products.

Despite the commercial success of yeast-derived products, the mechanisms at the base of YCW-pathogen interaction *in vitro* and *in vivo* are not clear. This is also a consequence of the heterogeneous nature and diversity, thus requiring several efforts for the optimization of the *in vitro* and *in vivo* assays to study the activity of these natural antimicrobial candidates. However, taking into account the *in vitro* studies, the trapping mechanism seems to be limited to some specific Gram-negative enteropathogens (*Salmonella* and *E. coli*), although several *in vivo* studies report their effect also on Gram-positive pathogens like clostridia. Furthermore, the complexity of the adhesion phenomenon makes it difficult to set up valid and reliable protocols for the *in vitro* testing of the antimicrobial effect of YCWs, and for better understanding the mechanisms that make YCWs favorable for some probiotic species and unfavorable for some pathogenic species.

The YCW products represent a sustainable solution to maintain the health of farm animals, thus representing a modern and ecological alternative to AGPs, although their effects on the target microorganisms are different. An appropriate management of animal nutrition in the farm, with a well-monitored usage of feed supplements, remains a valid strategy to counteract the emergence of feed/food safety hazards alongside the food chain from farm, thus supporting a more sustainable food production strategy.

The improvement of the usage of YCWs, either as AGPs or as treatment, requires a better understanding of the genetic and the biochemistry underlying the adhesion phenomenon. Understanding the mechanism at the base of this interaction could help on its super expression in specifically engineered or selected yeast strains. Moreover, YCW products also affect the gut microflora and improve the host immunity, thus resulting effective in preventing infections from pathogens whose adhesion to YCW could not be demonstrated *in vitro* (for example clostridia). In this perspective, combined use of appropriately setup *in vitro* methods and *in vivo* challenge tests (including scaled-up studies at intensive husbandry level) can be of pivotal importance in assessing the antimicrobial and growth-promoting effects of YCW products used as feed supplements in farm animal diet.

Disclosure Statement

No competing financial interests exist.

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