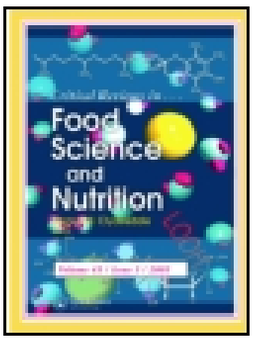

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REVIEW

Bacteriocinogenic probiotics as an integrated alternative to antibiotics in chicken production - why and how?

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ABSTRACT

The misuse of antibiotics in the livestock industry has played an important role in the spread of resistant superbugs with severe health implications for humans. With the recent ban on the use of antibiotics in poultry and poultry feed in Canada and the USA, poultry farmers will have to rely on the use of alternatives to antibiotics (such as feed acidifiers, antibodies, bacteriophages, antimicrobial peptides, prebiotics, and probiotics) to maintain the same productivity and health of their livestock. Of particular interest are bacteriocinogenic probiotics, that is, bacterial strains capable of producing bacteriocins that confer health benefits on the host. These bacterial strains have multiple promising features, such as the ability to attach to the host mucosa, colonize, proliferate, and produce advantageous products such as bacteriocins and short-chain fatty acids. These not only affect pathogenic colonization but improve poultry phenotype as well. Bacteriocins are antimicrobial peptides with multiple promising features such as being non-harmful for human and animal consumption, non-disruptive to the host microbiota eubiosis, non-cytotoxic, and non-carcinogenic. Therefore, bacteriocinogenic probiotics are at the forefront to be excellent candidates for effective replacements to antibiotics. While evidence of their safety and effectiveness is accumulating in vitro and in vivo in inhibiting pathogens while promoting animal health, their safety and history of use in livestock remains unclear and requires additional investigations. In the present paper, we review the safety assessment regulations and commercialization policies on existing and novel bacteriocinogenic and bacteriocin products intended to be used in poultry feed as an alternative to antibiotics.

KEYWORDS

Alternative to antibiotics; bacteriocin; livestock; poultry; regulations

Introduction

The poultry industry plays a significant economic role in both developing and developed countries and is the fastest-growing agricultural sub-sector (Alali and Hoface 2016; Mottet and Tempio 2017). Poultry meat and eggs are the most common animal source of protein consumed, and the second most-consumed meat globally (Alali and Hoface 2016; Mottet and Tempio 2017). It is expected that the global livestock sector will continue to grow, with the highest growth seen in the poultry industry with a 121% increased demand for meat and 65% for eggs (Mottet and Tempio 2017). Consumption of animal-sourced products provides a wide range of essential and micronutrients necessary for health, which would otherwise be difficult to obtain from consuming plants alone (Mottet and Tempio 2017). The safety of such products is paramount not just for consumers but for governmental regulatory agencies and producers. In the past, by moving away from free-range birds to confined systems together with the use of antibiotics as growth promoters, productivity increased and therefore allowed farmers to rise above the increased demand for poultry meat and products (Mottet and Tempio 2017). These changes also allowed poultry to be sold and marketed as a cheaper and complete protein source. While producers have managed the phenotype of the broilers to allow for maximum financial benefit, they are still struggling to maintain the health of the broiler and, by extension, the consumer. Indeed, poultry meat is implicated in numerous outbreaks and is the source of *Salmonella* and *Campylobacter* infections in humans (Alali and Hoface 2016). Presently, with the ban on the use of antibiotics as growth promoters and for prophylaxis, producers will have to change the way industry raises broilers by trying to mitigate the introduction and/or dissemination of poultry pathogens while maintaining the productivity of the past. The objective of this paper is to review the safety assessment regulations and commercialization policies on existing and novel bacteriocin products intended to be in poultry feed as an alternative to antibiotics. Recent meta-analyses and systematic reviews have substantiated the efficacy of bacteriocins and their producer bacteria in inhibiting pathogens in vitro, ex vivo, and in vivo. Although research

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This article has been corrected with minor changes. These changes do not impact the academic content of the article.

outcomes generally favor the utilization of bacteriocins in the treatment of infectious diseases, there are gaps in translating in vitro studies to animal studies due to the heterogenous nature of this category of antimicrobial peptides. Nowadays, bacteriocins are being recognized as an efficient alternative strategy to antibiotics, with three main distinctive features: (i) bacteriocins are ribosomally synthesized, (ii) are only toxic to bacteria, (iii) and have a relatively narrow killing spectrum (Hammami et al. 2013). All these futures have put bacteriocins to the forefront when searching for effective alternatives to antibiotics. Still, despite their antimicrobial potential and GRAS (Generally recognized as safe) status, few bacteriocins have reached commercial applications, mainly in food preservation. For decades, the lack of specific guidelines for legal approval of bacteriocin or bacteriocinogenic bacteria hindered their application in the veterinary and medical sectors (Soltani et al. 2021). As food and animal scientists have an important intermediary role between stakeholders and the livestock industry, we discuss bacteriocins and their producing bacteria from both scientific and regulatory aspects to promote the development of commercial veterinary applications as well as to encourage the growth of this promising field of research.

Antibiotics in poultry industry

Introduction

In 2016 in the United States, approximately 13.6 million kilograms of medically important antimicrobials approved for food-producing animals were sold and distributed (CVM 2017). Prophylaxis and growth promotion are the main reasons for supplying sub-therapeutic levels of antibiotics in the feed, water, and/or injected into muscles in the poultry industry (Marshall and Levy 2011; Pan and Yu 2013). Antibiotics are grouped into different classes based on their mode of action and further classified based on their importance to human medicine, as summarized in Table 1. The inhibitory mode of action of antibiotics differs depending on their class and target four major categories: (i) cell wall synthesis, (ii) protein synthesis, (iii) DNA replication, and (iv) folic acid metabolism. Antibiotic classes that inhibit cell wall synthesis are the β-lactams and glycopeptides (Kapoor, Saigal, and Elongavan 2017). Bacterial acquisition of resistance is inevitable, and numerous mechanisms exist that render antibiotics harmless such as changing the outer membrane permeability, modifying the target molecule, enzymatic inactivation of antibiotics, altering cell wall synthesis, mutation of enzymes, and ribosomal protection mechanisms, reviewed by (Kapoor, Saigal, and Elongavan 2017). Therefore, poultry and its products can be a source of introduction or dissemination of antibiotic-resistant pathogens (Holmes et al. 2016; Agyare et al. 2019). For instance, 5% of *Salmonella* tested in 2011 in the US was found to be resistant to five or more types of antibiotics (Center for Disease Control and Prevention 2015). This burden is evidenced in the US in 2014 and 2018 when multidrug-resistant *Salmonella,* linked to poultry products, was responsible for multistate outbreaks resulting in numerous cases of morbidity and mortality (Centers for Disease Control and Prevention 2018). In addition, the gut microbiota of poultry and other farm animals act as a large reservoir for resistance genes (Wang et al. 2019).

Impact of antibiotics on chicken gut microbiota

The most prevalent bacteria in chickens' gut are firmicutes, Bacteroidetes, and proteobacteria, accounting for more than 90% of the bacterial phyla (Pan and Yu 2013). The microbiota also contains several taxa that can cause illness in humans as well as the host. *Salmonella* spp., *Escherichia coli*, *Campylobacter* spp., and *Clostridia* spp. are examples of some of the pathogenic taxa that may be present (Oakley et al. 2014). Sex, age, feed additive, antibiotic, geography, litter type, and host genotype are just some of the factors that can influence the chicken's microbiota (Pan and Yu 2013; Li et al. 2017; Micciche et al. 2018). When the microbiota is balanced, pathogenic bacteria are inhibited due to several mechanisms involving both the host and microbiota, such as developed mucus layer, maintained intestinal integrity, competitive exclusion, and production of short-chain fatty acids (SCFAs) (Oakley et al. 2014). The microbiota eubiosis can be disrupted by antibiotics that may be used therapeutically, prophylactically, or for growth promotion, favoring certain bacterial classes over others (Li et al. 2017). Recently, Li et al. (2017) carried out a study to determine how the gut microbiota shifts in chickens that are infected with *Salmonella* and treated with enrofloxacin (a fluoroquinolone antibiotic). Authors reported that 25 genera were significantly enriched including the six abundant genera consisting of *Lactococcus, Bacillus, Burkholderia, Pseudomonas, Rhizobium,* and *Acinetobacter*, and 23 genera were significantly reduced in the medicated groups than in the control groups for the duration of the treatment period afterward the bacterial taxa did recover to normal levels (Li et al. 2017). In a similar study, avilamycin did not affect growth performance of chickens, while zinc bacitracin not only reduced the feed conversion ratio but also increased the richness and composition of the cecal microbiota by causing a reduction in the existing dominant species such as *Lactobacillus* (Crisol-Martínez et al. 2017).

Global regulations on the ban of antibiotics for growth promotion in animals

Antimicrobial consumption is expected to rise by almost 70% by 2030 (Mottet and Tempio 2017). While developed and some developing countries may have banned or are in the process of banning antibiotics in feed for growth promotion, the reality is that antibiotics are still used in the majority of countries in large quantities (Figure 1). Although colistin and carbapenem are the last resort antibiotics reserved for human medicine, resistance has started to appear in poultry and chicken meat (Food, Authority, and Efsa 2018). Due to increased global trading, food safety is a major priority for countries, and current food safety policies and regulations are starting to reflect that, particularly when

Table 1. Antibiotic categories, class, and route of administration in broiler chickens adapted from (Government of Canada 2018b).

Category of Importance in Human medicine	**Antimicrobial class**	Route of administration of Antimicrobials in broiler chickens	Antimicrobial
I Very High Importance	Carbapenems		
	Cephalosporins- 3rd or 4th generations	Subcutaneous or *in ovo* injections	Ceftiofur
	Fluoroquinolones		
	Glycopeptides		
	Glycylcyclines		
	Ketolides		
	Lipopeptides		
	Monobactams		
	Nitroimidazoles (metronidazole)		
	Oxazolidinones		
	Penicillin- -Lactamase inhibitor combinations		
	Polymyxins (colistin)		
	Therapeutic agents for tuberculosis		
II High Importance	Aminoglycosides (except topical agents)	Feed/ Water/Subcutaneous/ *in ovo* injections	Neomycin
			Apramycin
			Gentamicin
	Cephalosporins – the 1st and 2nd generations		
	Fusidic acid		
	Lincosamides	Feed/ Water	Lincomycin
			Lincomycin - spectinomycin
	Macrolides	Feed/ Water	Erythromycin
			Tylosin
	Penicillins		Amoxicillin
			Penicillin
	Quinolones (except fluoroquinolones)		Penicillin-streptomycin
	Streptogramins	Feed	Virginiamycin
	Trimethoprim-sulfamethoxazole		
III Medium importance	Aminocyclitols		
	Aminoglycosides (topical agents)		
	Bacitracins	Feed	Bacitracin
	Fosfomycin		
	Nitrofurans		
	Phenicols		
	Sulfonamides	Feed/ Water	Sulfamethazine
			Sulfaquinoxaline-pyrimethamine
	Tetracyclines	Feed/ Water	Chlortetracycline
			Oxytetracycline
			Tetracycline
	Trimethoprim		
IV Low importance	Flavophospholipols	Feed	Bambermycin
	Ionophores	Feed	Lasalocid
			Maduramicin
			Monensin
			Narasin
			Narasin-nicarbazin combination
			Salinomycin

it comes to reducing the spread of antibiotic resistance. In 2015, the World Health Organization (WHO) created a Global Action Plan on antimicrobial resistance (AMR), which called on member states to create their own action plans by 2017 (Snell 2019). The World Organization for Animal Health (OIE) and the Food and Agriculture Organization of the United Nations (FAO) jointly supported the resolutions in May and June of 2015, respectively (Snell 2019). The OIE released a report that stated that countries are still using antibiotics for growth promotion in 45 out of the 155 countries that provided data (Gulland 2019). Of these 45 countries, 18 are in the Americas, 14 are in Asia and Oceania, 10 are in Africa, two are in Europe, and one is in the Middle East (Gulland 2019). The names of the countries, however, were not released to continue to encourage participation in the ban on growth promoters (Gulland 2019). Of 45 countries, twelve used colistin as a growth promoter which is a last resort antibiotic and hence on the WHO's reserve list, 18 use bacitracin also on the reserve list, 17 use tylosin, and 15 use virginiamycin, both of which are on the WHO's "watch" list (Gulland 2019).

Europe

All the countries in the European Union (EU) follow regulations set by the EU regardless of whether they have or do not have stringent independent stewardship or monitoring systems in place. Starting in 2006, all antibiotics used as growth promoters were banned by the European Union (Congressinal Research Service n.d.). In October 2018, The EU released a press release statement regarding a new legislative policy to come into effect in 2022, which banned the use of antibiotics used prophylaxis in farming (Johnson 2018). Although the veterinary sale of antibiotics continues to decline according to the 2017 UK Veterinary Antibiotic Resistance and Sales Surveillance Report (UK-VARSS) report, it is apparent that the UK has not initiated any independent bans in the country unless forced to do so by the

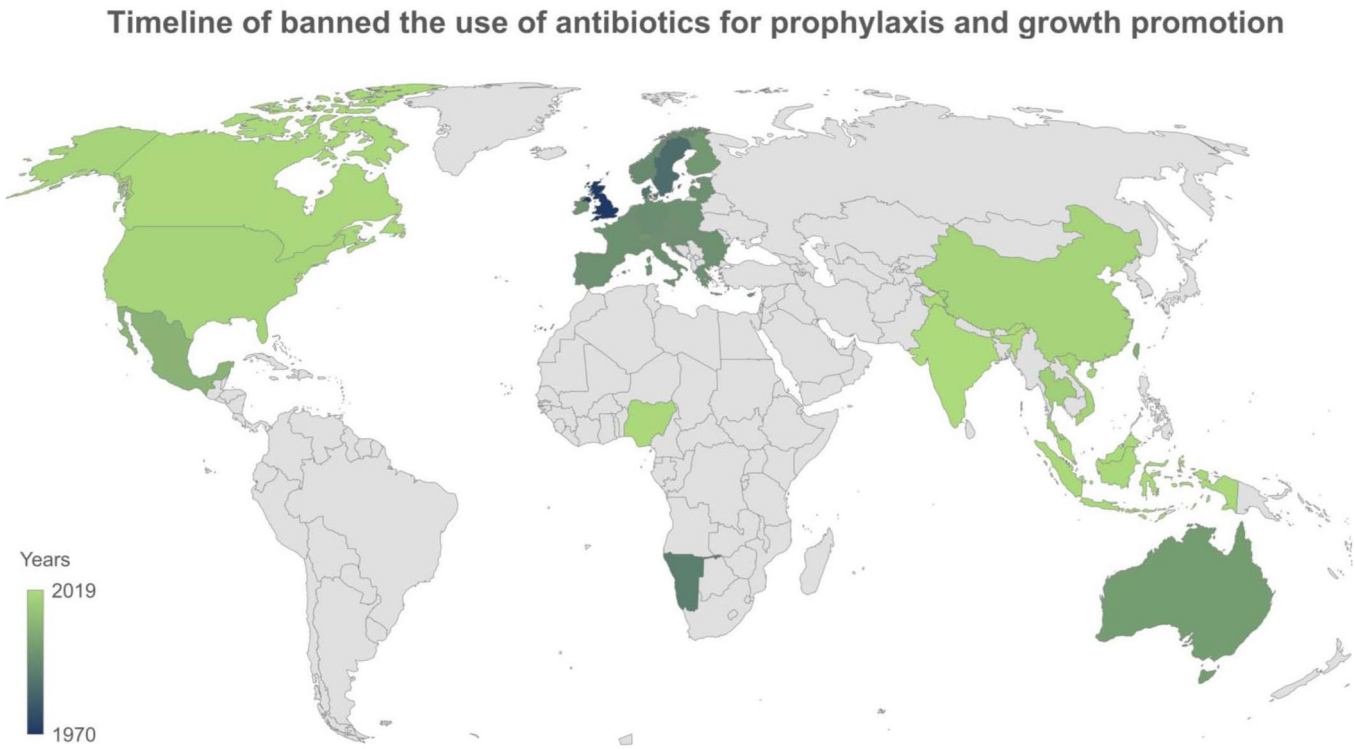

Figure 1. Timeline of banned the use of antibiotics for prophylaxis and growth promotion.

EU and with the current political uncertainty due to Brexit, there is fear that the UK might revert its bans after it exits the EU.

North America

In 2007, Mexico was first in North America to requires a veterinary prescription for the use of antibiotics in food-producing animals (Maron, Smith, and Nachman 2013). In 2010, the US Food and Drug Administration (FDA) put forward a strategy that involved the phasing out of medically important antimicrobials and bringing therapeutic antimicrobials under veterinary oversight. This was meant to be a voluntary endeavor by the industry (Food and Drug Administration 2013). This strategy was then drafted into a document in 2012, followed by the creation of a guidance document, a year later, that formally began the implementation of the strategy (Food and Drug Administration 2013). Then in 2017, the FDA announced that it would no longer allow medically important antibiotics to be used for growth promotion or feed efficiency (Food and Drug Administration 2018a) and in September 2018, the FDA released a five-year plan for supporting antimicrobial stewardship in veterinary settings of which one of the aims involves devising a strategy to bring all of the remaining medically important antimicrobials under veterinary oversight (Food and Drug Administration 2018a, 2018b). Similarly, in May 2014, the Chicken Farmers of Canada phased out the preventative use of Category 1 antibiotics, which was followed by Health Canada's Notice of Intent to strengthen veterinary oversight of antimicrobial use in April 2015 (The Chicken Farmers 2016; Government of Canada 2018a). This was soon followed by regulatory changes to the Food and Drug Regulations in 2017 (Government of Canada 2017). Lastly, Health Canada announced that starting December 2018, all medically important antimicrobials will require veterinarian prescription, thereby prohibiting their use as growth promoters and prophylactics (Government of Canada 2018b).

Asia

10 of the 11 WHO's South-East Asia region countries have their own National Action Plans, and WHO's 11 of the 37 Western Pacific region countries have plans with 5 having draft plans prepared (Snell 2019). For instance, China announced in 2015 and 2016 the creation and implementation of two National Action Plans on AMR with primary goals are to reduce in half the use of antibiotics in animal agriculture by prescription, to phase out the critically important antibiotics for human health as well as those that have the potential to cross-transmit AMR, and those that are used as growth promoters (Coller 2019; Wu 2019). This is ambitious as it was estimated that in 2013, 84,240 tons of antibiotics had been used in animal production in China (Yang et al. 2019). Likewise, India has developed a National Action Plan in order to restrict and phase out the use of antibiotics for non-therapeutic purposes in animals (Coller 2019). In August 2019, the Food Safety and Standards Authority of India (FSSAI) announced the ban on the use of colistin by drafting Food Safety and Standards (Contaminants, Toxins and Residues) Amendment Regulations, 2019 (Food Safety and Standards Authority of India 2019; Walia et al. 2019). Similarly, Sri Lanka has

created a National Action Plan aligned with WHO's Global Action Plans for Combating Antimicrobial Resistance 2017–2022, which among many aims, will reduce and eventually phase out antibiotic growth promoters in animal feed and poultry production (Sri Lanka 2017; Ralte 2018).

Australia

Similar to New Zealand, Australia has one of the lowest use of antibiotics in food-producing animals, lowest levels of AMR, and strict regulations on the use of chemicals in the world (Ludlow 2010). The Australian Working Party on Antibiotics (WPA) has never authorized fluoroquinolones to be used in livestock, but other growth-promoting antibiotics were allowed (Ludlow 2010). To date, gentamicin is banned from use, 3rd generation cephalosporins have restricted use, and cefquinome has not been registered for livestock use (Ludlow 2010).

Africa

In 1991, Namibia was the first African country to ban antibiotics as well as hormones for growth promotion in its beef industry (WHO 2017). Nigeria banned the use of antibiotics as growth promoters in animal feed in 2018 as a result of a rise in AMR deaths in the country (Coller 2019).

Bacteriocins as promising alternatives for the poultry industry

Introduction

Bacteriocins are a heterogeneous family of small proteinaceous molecules with a relatively narrow bacteriostatic or bactericidal spectrum of activity and mechanisms of action (Cavera et al. 2015; Chikindas et al. 2018). The anti-infective potential of bacteriocins for inhibiting pathogens has been shown in various food matrices, including cheese, meat and vegetables (Hammami, Fliss, and Corsetti 2019), but also in therapeutic practice (Hammami et al. 2013). Three main features distinguish the majority of bacteriocins from conventional antibiotics: bacteriocins are ribosomally synthesized, are only toxic to bacteria and have a relatively narrow killing spectrum (Hammami et al. 2013). All theses futures have put bacteriocins to the forefront when searching for effective alternatives to antibiotics.

Bacteriocins make up a highly diverse family of proteins in terms of size, microbial target, mode of action and release and mechanism of immunity and can be divided into two broad groups: those produced by Gram-negative bacteria and those produced by Gram-positive bacteria, with the latter being more abundant and more diverse (Hammami et al. 2010). According to their size and post-translational modification, bacteriocins are categorized in four different classes (for review (Cavera et al. 2015)): Class I involves bacteriocins that undergo post-translational modifications, such as the lantibiotics (Józefiak and Sip 2013; Cavera et al. 2015). These bacteriocins contain lanthionine in their structure, have a molecular mass of under 5 kDa, and are thermostable, membrane-active peptides (Józefiak and Sip 2013). Class II bacteriocins, the non-lantibiotics, involve no or minimal post-translational modifications and are similar to class I bacteriocins in that they are also thermostable and membrane-active peptides (Józefiak and Sip 2013; Cavera et al. 2015). Class II bacteriocins have a molecular mass of under 13 kDa, and the presence of a Gly-Gly sequence present in the precursory peptide is characteristic of class II bacteriocins (Józefiak and Sip 2013). Class II bacteriocins are further sub-classified; IIa- pediocin like bacteriocins, IIb-dipeptide bacteriocins, IIc- sec-dependent bacteriocins, and IId- bacteriocins (Jozefiak et al. 2013). Class III bacteriocins are non-membrane active peptides that have a molecular mass of higher than 30 kDa and are thermolabile (Jozefiak et al. 2013). Lastly, class IV bacteriocins are those that form protein-lipid or protein-carbohydrate complexes (Jozefiak et al. 2013).

The bacteriocin action starts with entry into the target cell by recognizing specific cell surface receptors. Then, Microbial cell killing occurs through various mechanisms: formation of ion-permeable channels in the cytoplasmic membrane, nonspecific degradation of cellular DNA, inhibition of protein synthesis through the specific cleavage of 16 s rRNA, or by cell lysis resulting from inhibition of peptidoglycan synthesis (Vriezen et al., 2009). Figure 2 provides an overview of the targets of antibiotics and bacteriocins. For instance, bacteriocins, including Lactococcin A, can bind to membrane-bound glucose and/or mannose phosphotransferase system (man-PTS), which causes an efflux of potassium ions (K^+), disrupting the membrane potential and ultimately the membrane (Cavera et al. 2015). Lantibiotics, such as nisin A, attach to lipid II, which prevents the transport of the precursory peptidoglycan molecules from the cytoplasm to the cell membrane, thereby inhibiting cell wall synthesis (Chugunov et al. 2013; Cavera et al. 2015). Comparatively, colicins E3, E4 and E6 and cloacin DF13 target protein synthesis pathways, leading to malformed proteins and, ultimately, cell death (Cavera et al. 2015). Other Gram-negative bacteriocins, such as Microcin B17, competitively inhibit the ATPase active site on the DNA gyrase, specifically the GyrB subunit, which causes binding and prevention of the decatenation of replicating DNA (Cavera et al. 2015).

The mechanisms related to the target-specificity of bacteriocins are poorly investigated. The Man-PTS protein complex can be categorized into multiple phylogenetic groups; however, only members from phylogenetic group I were reported to serve as receptors for class II bacteriocins (Tymoszewska et al. 2017). Although an increasing number of class II bacteriocins were shown to recognize and bind specifically Man-PTS in the target hosts, these bacteriocins exhibit distinct spectra of inhibitory activity, being highly potent against only single species or a wider range of bacteria (Tymoszewska, Walczak, and Aleksandrzak-Piekarczyk 2020). Besides, the Man-PTS-targeting bacteriocins also differ significantly in their amino acid sequence, suggesting

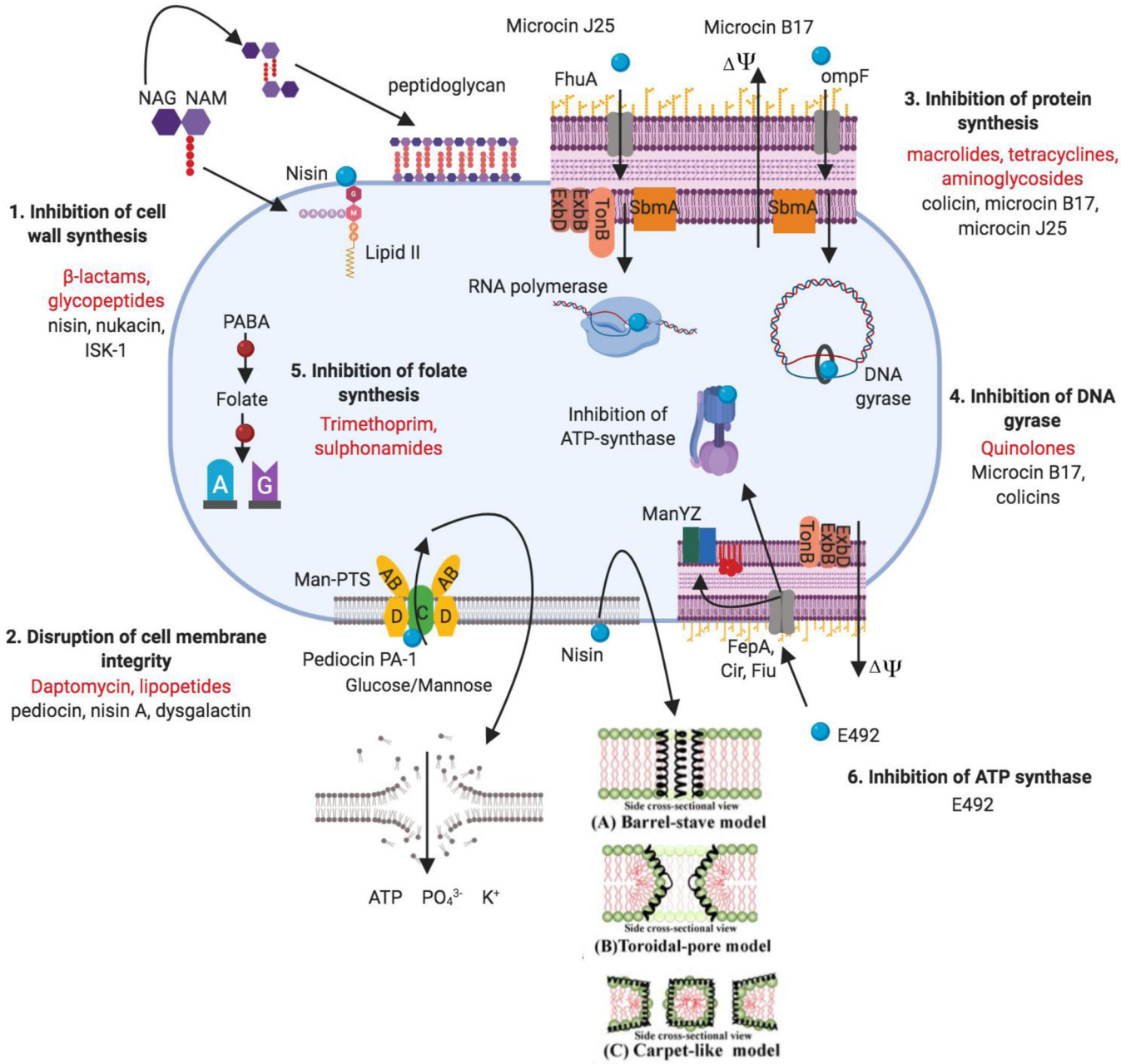

Figure 2. Overview of the similarity in targets between bacteriocins and antibiotics.

distinct modes of interaction with Man-PTS (Tymoszewska, Walczak, and Aleksandrzak-Piekarczyk 2020).

The inhibitory spectra of bacteriocins are generally relatively narrow and directed primarily against species closely related to the producing strain, with the vast majority of known bacteriocins are products of Gram-positive bacteria (Hammami et al. 2013). Known producers among Gram-negative bacteria are few, and their bacteriocins are less diverse (Hammami et al. 2013). Despite the need to discover additional bacteriocins, the already described ones demonstrated successful inhibition of many poultry-related pathogens. Indeed, multiple bacteriocins were reported effective against *Salmonella*, *Clostridium perfringens*, and *Campylobacter* (Hammami et al. 2013), three leading pathogens that cause foodborne illnesses and linked explicitly to poultry products (Hofacre, Smith, and Mathis 2018; Liu et al. 2018). For example, several potent anti-*Campylobacter* bacteriocins produced by commensal bacteria isolated from the chicken intestine have been purified and characterized, including bacillocin B602 from *P. polymyxa*, E50-52 and E-760 from *Enterococcus* spp., OR-7 and L-1077 from *L. salivarius*, reviewed in (Hammami et al. 2013). Combining bacteriocins or their producer bacteria could also be an effective strategy to target multiple pathogens simultaneously in chicken GIT. As both pathogens are colonizers of the chicken gut and cause foodborne outbreaks, it is logical to focus on creating a healthy intestinal environment to mitigate the colonization and proliferation of such pathogenic strains. In this regard, many bacteriocins were shown to minimally disrupt the gut microbiota in a mouse model (Umu et al. 2016).

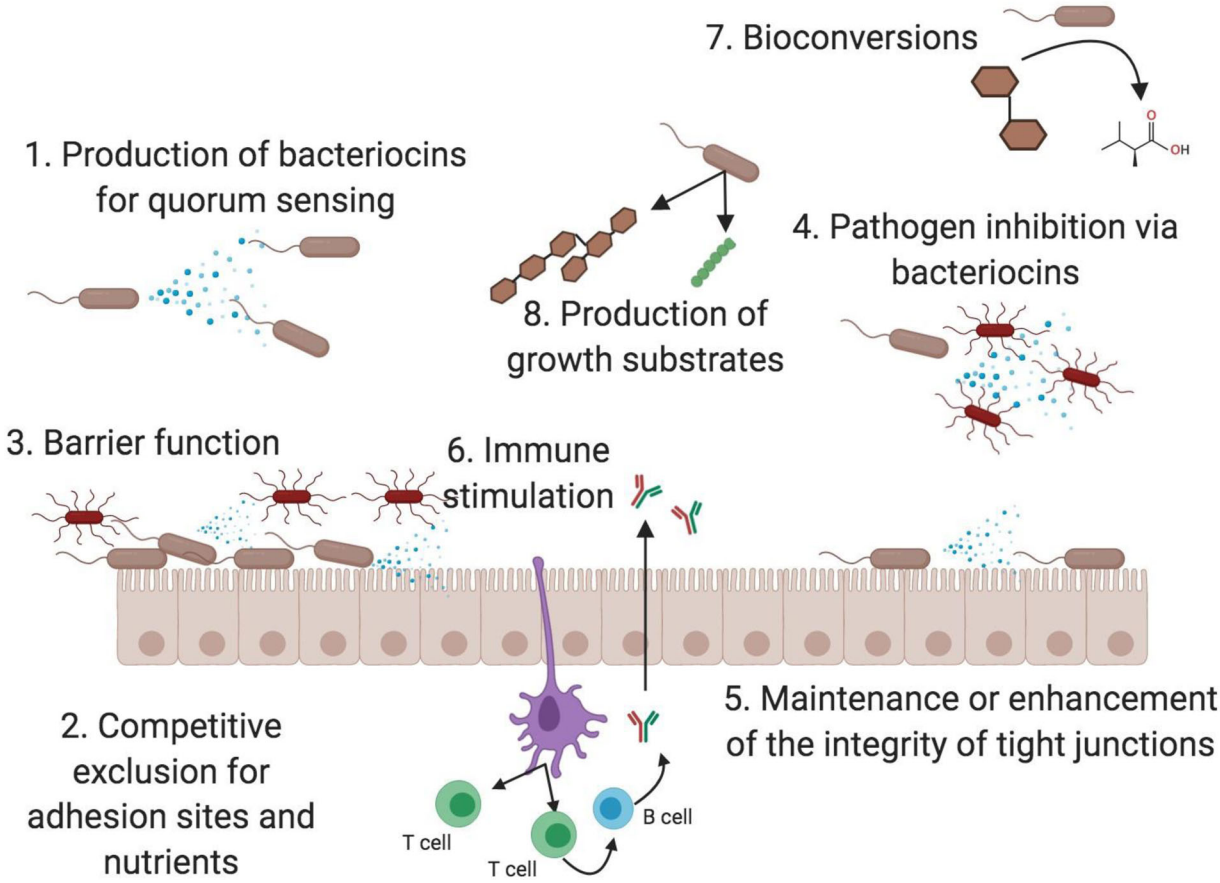

Figure 3. Overview of the mechanisms of interaction of bacteriocinogenic strains in the intestinal tract. Adapted from (O'Toole and Cooney 2008).

Bacteriocins and bacteriocinogenic bacteria in the poultry gut

Bacteriocins and bacteriocinogenic strains have been widely studied in their ability to not only control poultry and human pathogens but also to determine their effects on poultry phenotype, such as improved meat quality, carcass weight, and weight gain by the birds. The bacteriocins that have been isolated from the poultry gastrointestinal tracts show a broad spectrum of activity (Józefiak and Sip 2013). Bacteriocins and bacteriocinogenic strains allow GIT colonization via mechanisms such as competitive exclusion and quorum sensing (Figure 3). Quorum sensing employed by bacteriocinogenic strains may be used more like a signaling and defensive strategy rather than a killing strategy, according to Chikindas et al. (2018). Therefore, bacteriocins could be released in a low concentration to inhibit biofilm formation by intruding strains by the inhibition of quorum sensing (Chikindas et al. 2018). Bacteriocins may also be released as a quorum-sensing molecule itself to identify similar strains in a new niche prior to colonization.

Competitive exclusion (CE) is a mechanism employed by probiotic strains whereby they compete against commensal bacteria in the niche environment for common adhesion site along the mucosa, and for nutritional sources (Chichlowski et al. 2007; Wan et al. 2019). The advantage of being involved in CE is the ability to modulate the microbiota by excluding the presence of specific bacterial strains that might share the same adhesion sites or by producing antimicrobial compounds such as SCFAs or bacteriocins, making the environment inhabitable for their competitors (Wan et al. 2019). The inhibition of pathogens in the GIT is usually through this mechanism (Wan et al. 2019). Besides being involved in competitive exclusion and quorum sensing, bacteriocinogenic strains also can affect the histology and ultrastructure of the GIT due to regulation of mucus production and secretion (Chichlowski et al. 2007). Furthermore, the bacteriocinogenic strains can maintain or enhance the integrity of the tight junctions during pathogenic infections or inflammatory conditions (Chichlowski et al. 2007).

Bacteriocins in poultry feed

Due to the specific characteristics of bacteriocins, the direct addition of bacteriocins to poultry feed could have beneficial effects by not only boosting the existing concentrations of bacteriocins but also by allowing the bacteriocinogenic strains to allow continued colonization even in the presence of pathogens (Józefiak and Sip 2013). As a poultry producer, maximal profits must be reaped from the product that is invested in. This requirement translates to the need for an improved feed conversion ratio (FCR) and hence improved weight gain. FCR is the weight of the feed, provided over the lifetime of the animal, that allows an animal to put on weight

and is a measure of feed digestibility, that is, the amount of feed absorbed and the amount of nutrients that are available for the growth and reproduction of the animal (Vieco-Saiz et al. 2019). Feed conversion ratio may also impact the quality of meat and carcass weight. Having healthy flocks that lack subclinical or clinical symptoms is also a key factor in maximizing profits. When added to feed, bacteriocins should optimize the nutritive value of the feed due to its positive benefits that arise when ingested. The concentration of the added bacteriocins should be enough to mount beneficial effects as well as its survivability of the harsh environment of the GIT. Previously, antibiotics were utilized as a growth promoter to increase bird weight and FCR. It was also used therapeutically or prophylactically to ensure birds were free of harmful pathogens such as *Clostridium perfringens*, *Campylobacter spp.* and *Salmonella spp*. With the bans in place on the use of antibiotics for prophylactic and/or growth promotion purposes, producers need alternatives that will function in much the same way as antibiotics once did. Bacteriocins or bacteriocinogenic strains fit these criteria.

It is well known that the gut microbiota competes with the host for available nutrients and may have a negative effect on the host (Józefiak and Sip 2013). Loss of nutrients coupled with subclinical infections from pathogens, is translated as weak growth in the birds. The products of digestion and fermentation by gut bacteria is the production of SCFAs, such as acetic acid, butyric acid, and propionic acid, which have a favorable effect on the host as these acids can have a bactericidal or bacteriostatic effect on pathogens. The targets for bacteriocins should be the latter portion of the GIT, mainly the ileum and ceca. This is because these anatomical regions are colonized by anaerobic fermenting bacteria and have a diverse population of bacteria compared to the upper gut. This region is also where pathogens such as *Salmonella* like to colonize. Bacteriocins or bacteriocinogenic strains, via competitive exclusion, would be able to prevent colonization by pathogenic strains as well as help in the elimination of existing colonized pathogens. Quorum sensing would allow the coordinated production of bacteriocins to allow an increase in concentration and thereby help with the elimination of pathogens, especially if the bacteriocinogenic strain will also produce SCFAs. This dual mode of action would be very effective in the elimination and or prevention of infections while at the same time helping with the performance of the host by helping with weight gain by effective FCR. (Park et al. 2016) reported that *Lactobacillus spp.*, a commonly known bacteriocinogenic strain, was able to improve the quality of poultry meat in terms of improved tenderness, appearance, texture, and juiciness. Similarly, the addition of LAB has also been known to reduce the cholesterol content and increase the weight of poultry meat in a strain-specific manner (Vieco-Saiz et al. 2019). For example, *Lb. delbrueckii*, *Lb. acidophilus*, *Lb. casei*, *Lb. agilis*, *Lb. salivarius, Lb. fermentum*, and *Lb. ingluviei* were found to be responsible for weight gain (Vieco-Saiz et al. 2019). Bird performance was improved due to the ability of LAB to ferment food and produce SCFAs as well as the production of digestive enzymes, and B- vitamins (Vieco-Saiz et al. 2019). This allowed the metabolism of food nutrients to be better utilized by the gut bacteria avoiding the usual competition with the host for nutrients.

The in vivo stability and appropriate delivery route remain important questions for bacteriocins and their producer bacteria. Particularly, loss of peptide activity in vivo due to proteolytic enzymes in the gut was reported in multiple studies, reviewed in (Soltani et al. 2021). For example, nisin, a class I lantibiotic, shows promising activities in vitro and animal models against numerous clinically relevant bacteria strains. However, its inherent chemical instability and poor solubility at pH 7.0 place a technological hurdle before its application as a therapeutic agent (Hammami et al. 2013). While *L. lactis* UL719, a nisin Z producer, was able to survive these GIT stressful conditions, to keep the ability to produce its bacteriocin, purified nisin Z was highly sensitive to digestive enzymes, especially pancreatin (Le Lay 2015). Likewise, similar results were observed for pediocin PA1, a class II bacteriocin, using the TNO dynamic model of the stomach and small intestine (TIM-1) (Kheadr et al. 2010). Recently, we provided evidence of the degradation of microcin J25 (MccJ25) in simulated human duodenal conditions (TIM-1) despite its highly stable lasso structure (Naimi et al. 2018). While MccJ25 was not affected by acidic pH, pepsin, and lipase in gastric conditions, partial degradation of MccJ25 was detected in the duodenal compartment due to pancreatic elastase I (Naimi et al. 2018). Together, these findings portend that bacteriocins may be considered safe products for various potential applications as an alternative to antibiotics. However, since bacteriocins are likely to be degraded by proteases in the small intestine, the ingestion of bacteriocin-producing strains should be a preferable oral route to ingestion of the purified bacteriocin. We believe that the use of the producer strain itself offers several advantages over purified bacteriocins (Fernandez et al. 2013). Alternatively, bacteriocin concentrations higher than the MIC values or peptide protection by gastrointestinal resistant encapsulation materials are needed to maintain the antibacterial activity in the GIT (Fernandez et al. 2013).

Bacteriocins in the feed should be able to remain stable during the production of the feed due to the use of high temperature and pressure. Some researchers have shown the continued efficacy of bacteriocins even when they have been encapsulated or lyophilized to prevent the digestion and absorption of the bacteriocins in the upper segment of the GIT (Józefiak and Sip 2013). However, it may be financially reasonable to focus more on bacteriocinogenic strains instead of using pure bacteriocins due to ease of production, stability, and dosage requirements. In addition, formulations containing more than one bacteriocinogenic strain would be beneficial due to the varied mode of action of the bacteriocins involved.

Bacteriocins in pathogen clearance in poultry GIT

The efficacy of bacteriocins in pathogen clearance in poultry is summarized in Table 2. *C. jejuni* is a human pathogen that causes campylobacteriosis and spreads to humans via contaminated chicken carcasses and/or contaminated poultry meat due

Table 2. Summary of the efficacy of bacteriocins in pathogen clearance and poultry phenotype.

Bacteriocin	Producer	Origin	Target microorganism	Growth Performance	References
Plantarcin	*L. plantarum* DM 69	Yoghurt (Buffalo milk)	*S. enterica*		(Mohanty et al. 2019)
Pediocin A	*Pediococcus pentosaceus*	Cucumber fermentation	*C. perfringens* type A	Strong inhibitory capacities, Weight recovery at similar levels of healthy birds	(Grilli et al. 2009)
Divercin	*Carnobacterium divergens* AS7	Fish	*C. perfringens*	Improved growth performance, nutrient retention, intestinal histomorphology, balance of gastrointestinal microbiota	(Jozefiak et al. 2011a, 2011b, 2012)
Nisin	*Lactococcus lactis subsp. Lactis*			Improved body weight gain and feed conversion ratio	(Kieronczyk et al. 2017)
Perfrin	*netB-positive C. perfringens*	Chicken with necrotic enteritis	*C. perfringens* Type A		(Timbermont et al. 2014)
Dietary nisin	*Lactococcus lactis* subsp. *lactis*			Improved body weight gain, Increases feed conversion	(Józefiak et al. 2013)
AP 216, AP 45	*E. faecalis*	Pig feces	*C. perfringens*	Antimicrobial activity demonstrated	(Han et al. 2014)
FK22	*L. salivarius* K7	Chicken intestine	Gram positive bacteria		(Sakpuaram et al. 2006)
OR-7	*Lactobacillus salivarius* NRRL B-30514	Cecal contents from healthy commercial broiler chickens	*C. jejuni*	Reduction in colonization by at least 1 million-fold	(Stern et al. 2006)
L-1077	*Lactobacillus salivarius* 1077 (NRRL B-50053)	Poultry intestinal materials	*C. jejuni*		(Svetoch et al. 2011)
SMXD51	*Lactobacillus salivarius* SMXD51	Chicken ceca	*C. jejuni, C. coli*		(Messaoudi et al. 2011, 2013)
Enterocin S37	*Enterococcus faecalis* S37	Chicken feces	*Listeria monocytogenes EGDe, L. innocua, E. faecalis* JH2-2, *Lactobacillus brevis* F145		(Drider et al. 2010)
Albusin B	*Ruminococcus albus*	Rumen	Commensal *Salmonella* and *Enterococcus*	Improved gut barrier function, improved growth performance, increased intestinal absorption of protein and glucose, elevated fecal lactobacilli and reduced enterococcus and salmonella counts, modulated lipid metabolism, activated systemic antioxidant defense	(Wang et al. 2011, 2013)

to gut contents (Svetoch and Stern 2010). (Svetoch and Stern 2010) reported the elimination of *Campylobacter jejuni* from infected chicks when purified bacteriocins were provided in feed or water. Similarly, chickens treated with bacteriocins such as pediocin A from *Ped. pentosaceus*, divercin from *Carnobacterium divergens* AS7, and plantaricin from *Lb. plantarum* F1, it was found that the bacteriocins were able to improve health and allow the chickens to gain weight even after they had been infected by *C. perfringens* and *E. coli*, respectively (Vieco-Saiz et al. 2019). *C. perfringens*, a causative agent of necrotic enteritis, is currently the major poultry pathogen of concern due to a high number of animal mortality and the resulting production losses (Ben Lagha et al. 2017). Production of bacteriocins that target pathogens is not limited to the LAB. In fact, many *Bacillus* spp. have been shown to produce bacteriocins that have the capability to significantly lower the *C. perfringens* counts in the chicken intestines (Vieco-Saiz et al. 2019). Bacteriocins have also successfully shown to control *Salmonella.* Reuterin, an antimicrobial substance, produced by both *Lactobacillus reuteri* ATCC 55730 and *L. reuteri* L22, was found to be able to significantly reduce the growth of *Salmonella* pullorum ATCC 9120 in MRS broth and *L. reuteri* ATCC 55730 was also able to increase the survival rate of a chick infected by *Salmonella pullorum* (Zhang et al., 2012). Similarly, Kim et al. (2015) were able to show that kimchi and broiler chicken isolated lactic acid bacteria were able to produce bacteriocin-like substances, which had strong antimicrobial activity against *Salmonella* Enteritidis, Heidelberg, Newport and Typhimurium (Kim et al. 2015).

Food safety regulations and policies for commercial use of bacteriocins

Introduction

Bacteriocinogenic probiotics are at the forefront as the next best alternative to the use of antibiotics in poultry. However, even though microorganisms have been used for centuries for the preservation of food, not many bacteriocins or novel bacteriocinogenic strains have been commercialized apart from nisin and pediocin as food preservatives. Commercialization of bacteriocins or their producing strains have different policies not only in different countries but also for their intended use. However, the regulations that exist for food cultures would also, for the most part, apply for the use of microorganisms as feed additives in animals. Regardless of whether the intended use is in humans or animals, the first step that needs to be taken is to identify a potential strain or biological compound at the strain level. The nomenclature and proprietary name of the probiotic strain or bacteriocin, its mode of action, and its composition are to be clearly stated. This process is then followed by characterization and development of a safety profile, which includes but is not limited to the capacity to produce toxins, presence of virulence factors, and/or transmissible mobile

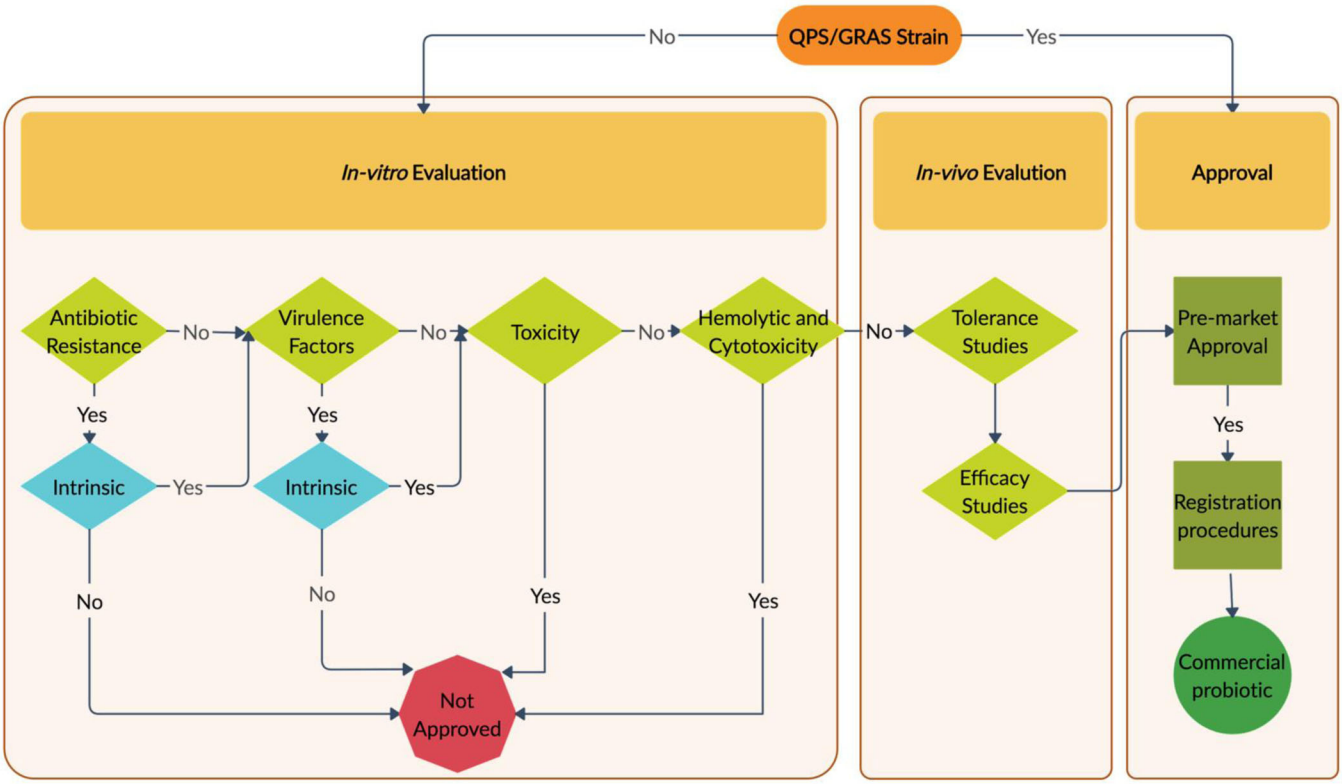

Figure 4. Illustration of the current steps required for the commercialization of bacteriocinogenic probiotics.

genetic elements, antibiotic production and resistance (Anadón, Rosa Martínez-Larrañaga, and Aranzazu Martínez 2006). The strain or bacteriocins' stability and any incompatibilities with other feed ingredients should also be stated (Anadón, Rosa Martínez-Larrañaga, and Aranzazu Martínez 2006). This is particularly the case when a novel strain is being put forward to be commercialized, as stringent documentation is required for its history of safe use. It is also important to determine whether that strain produces metabolites and the effect of such chemicals as they may either help preserve the food or render it toxic. Figure 4 illustrates the current steps required for the commercialization of bacteriocinogenic probiotics.

Regulations and policies on the commercialization of novel and existing microorganisms used as feed additives

European Union

The European Food Safety Agency (EFSA) utilizes two references when it comes to making decisions on food culture safety, the Qualified Presumption of Safety (QPS) and the Inventory of Microorganisms with Technological Beneficial Use from the International Dairy Federation (IDF) (Laulund et al. 2017). The QPS is a list of species and strains that EFSA refers to when evaluating the safety of an additive that is microbial in nature, thereby allowing the additive to be fast-tracked for approval of commercial use. It covers all risk assessment for microorganisms for humans, animals, and environmental use, presumed safe, and therefore do not require specific studies showing its safety assessment (Laulund et al. 2017; Rychen et al. 2018). The Panel on Biological Hazards (BIOHAZ) at EFSA, determines the safety of the biological agent by looking at the documentation on the taxonomy, existing knowledge of the strain, any safety concerns and depending on the species, the end use of the agent (Laulund et al. 2017). QPS applies to the regulation of the use of microorganisms as feed additives but not when used for fermentation of foods (Anadón, Rosa Martínez-Larrañaga, and Aranzazu Martínez 2006). The QPS can be considered to be the equivalent of the GRAS status used in the USA with some differences such as the application of GRAS for all ingredients, whereas QPS applies to microorganisms only (Laulund et al. 2017). Another major difference between GRAS and QPS is that the GRAS status applies to the strain and the particular food product, whereas QPS applies to the taxonomic unit of a species and not the product that contains it (Laulund et al. 2017).

In the European Union, mainly Gram-positive bacteria that belong to *Bacillus* (*B. licheniformis*, *B. subtilis*), *Enterococcus* (*E. faecium*), *Lactobacillus* (*L. acidophilus*, *L. casei*, *L. farciminis*, *L. plantarum*, *L. rhamnosus*), *Pediococcus* (*P. acidilactici*), and *Streptococcus* (*S. infantarius*) are used; however, Enterococci was excluded by the QPS list in 2014 (Laulund et al. 2017). If a microorganism is not found on the QPS list, it does not mean that they are considered unsafe; instead, it might be that EFSA has not evaluated the species and its full safety yet (Laulund et al. 2017). It should be noted that which microorganisms require pre-market approval and safety evaluation is only authorized by the EU Commission (Laulund et al. 2017).

Canada

Feeds v. Drugs. In Canada, products intended for livestock can be either regulated as "Feed" or as "Drugs" depending on the intended purpose of the product. The intended purpose is defined as the 'desired effect', that is, what is the main intention that is to be achieved by the administration of the product (Government of Canada 2019). Depending on the product, its ingredient(s) and application conditions can also provide information as to the desired effect of the product (Government of Canada 2019). Veterinary drugs are regulated under The Food and Drugs Act and Regulations administered by Health Canada, which ensures the safety, effectiveness, and quality of the product (Government of Canada 2019). The livestock feeds, on the other hand, are regulated under the Feeds Act and Regulations, administered by the Canadian Food Inspection Agency (CFIA), whose aim is to ensure that the feeds, domestic and imported, are safe, effective, and labeled correctly (Government of Canada 2019).

In this Act, a "feed" is defined as, "any substance or mixture of substances containing amino acids, antioxidants, carbohydrates, condiments, enzymes, fats, minerals, non-protein nitrogen products, proteins or vitamins, or pelletizing, coloring, foaming or flavoring agents and any other substance manufactured, sold or represented for use: i. for consumption by livestock; ii. For providing the nutritional requirements of livestock, or; iii. For the purpose of preventing or correcting nutritional disorders of livestock, iv. or any substance for use in any such substance or mixture of substances" (Government of Canada 2019). A "drug" is defined as "any substance or mixture of substances manufactured, sold or represented for use in i. the diagnosis, treatment, mitigation or prevention of a disease, disorder, abnormal physiological state, or its symptoms in human beings or animals, ii. Restoring, correcting or modifying organic functions in human beings or animals, or iii. disinfection in premises in which food is manufactured, prepared or kept" (Government of Canada 2019).

As mentioned above, the intended purpose of the product dictates whether the product is classified as a feed ingredient or a drug even though some ingredients can be both therapeutic and nutritional due to their function and effect on the organism or their purpose in the formulation (Government of Canada 2019). In such a case, the therapeutic effect outweighs the nutritional effect, and the product will be classified as a drug. Similarly, a multiple-ingredient product would be considered a drug if one or more ingredients in its composition have a therapeutic effect (Government of Canada 2019).

A therapeutic product claim or purpose refers to the treatment of "a disease, disorder or abnormal physical state; or treatment, mitigation of its symptoms; or the modification of an organic function (such as digestion)" and as such can only be made for drugs and not for feeds (Government of Canada 2019). Conversely, feeds are not allowed to have therapeutic claims or purposes, but they can act as carriers for therapeutic products (Government of Canada 2019). Feeds are intended to be used as part of a feeding program whose purpose is to allow for the growth and maintenance of healthy livestock for a reasonably short amount of time (duration of the livestock) (Government of Canada 2019). Feed ingredients are not limited to nutrients but can also include non-nutritive products such as flavors, pellet binders, preservatives, anti-caking agents and other products that are added to allow for safe storage, palatability of feeds, or even help with the manufacturing process (Government of Canada 2019).

Bacteriocins: Feeds or drugs. Based on the above information, currently, bacteriocins would be classified as drugs instead of feed additives, and this is different from how they would be classified in the EU, as feed, not drugs. The 'drugs' label designation is primarily due to two reasons. First, due to their mode of action in the intestines, that is, their interaction with the gut microbiota, which can be both therapeutically and/or prophylactically. Therapeutically due to the bactericidal effect on the pathogens and prophylactically by ensuring the colonization of the native microbiota in the intestinal tract making it difficult for pathogenic bacteria to colonize the niche. Appendix E-2 Viable microbial products (VMPs) of the guidance document on the classification of veterinary drugs and livestock feeds, clearly states that depending on the properties of the microbial strains, such as the production of bacteriocins or antimicrobial peptides, the product may be classified as a drug (Government of Canada 2019). VMPs are live microorganisms, individual or multiple strains, that have been incorporated into feeds or other dosage forms and whose purpose is to have a beneficial effect in the target organism (Government of Canada 2019). It appears that some VMPs fall under a new label of "Gut modifier (gastrointestinal modifier)" to classify those VMPs whose mode of action involves gut microbiota modification instead of the practice of classifying them as veterinary drugs (Canadian Food Inspection Agency 2019). This new category will also allow additional claims of nutritional or production/performance as regulated under the Feeds Act and Regulations (Canadian Food Inspection Agency 2019).

USA

The FDA regulates the use of bacteriocins and bacteriocinogenic strains under the Federal Food, Drug, and Cosmetic Act (FFDCA) where they are regulated as food ingredients because under the FFDCA Act; food is defined as, "articles used for food or drink for man or other animals … " (Fields 1996; Food and Drug Administration 2019). The FFDCA is also responsible for granting the GRAS status based on either the historic safe use of the ingredient in food or scientific data and, as such, are exempt from the pre-market approval, which is mandatory otherwise (Fields 1996). FDA's Center for Veterinary Medicine (CVM) is responsible for animal feed product regulation.

Similar to Canada, bacteriocins would be classified as 'new animal drug' rather than feed ingredients and fall under the regulation of the FDA's CVM (Fields 1996). New animal drugs are defined under section 201(w) as "any drug intended for use in animals", which does not have the GRAS

status (Fields 1996). Pre-market approval is also required for those ingredients not granted the GRAS status and if it is expected to become a component of animal food (Fields 1996; Food and Drug Administration 2019). A prerequisite of the approval of the food additive is the approval of a food additive petition which must meet the criteria listed under Title 21 CFR 570 and 571 of the FFDCA Act and consists of information required on human and target animal safety, impact on the environment, manufacturing details, proposed labeling and regulations etc. (Fields 1996; Food and Drug Administration 2019).

Criteria for selection of bacteriocinogenic bacteria as feed additives

Strain identification

The document should contain the complete taxonomy, nomenclature, origin of the strain or biological compounds such as bacteriocins, hereby referred to as the 'additive', any genetic modifications that have occurred, and methods for the control of the strains (Anadón, Rosa Martínez-Larrañaga, and Aranzazu Martínez 2006; Rychen et al. 2018). Additive identity and safety assessment are conventionally performed using traditional phenotypic culture-based methods or molecular methods such as Pulse Field Gel Electrophoresis (PFGE), however with the advancements in molecular sequencing methods mainly Whole-genome sequencing (WGS), using new and advanced molecular methods are now becoming the ideal approach for not only strain identification but also to correctly interpret and confirm the results of antibiotic resistance and virulence factors tests (Laulund et al. 2017; Rychen et al. 2018). Prior to using WGS, a complete taxonomic characterization must already be performed (Laulund et al. 2017).

Efficacy studies

Efficacy studies of the additive in question must be performed in the target species, be based on at least three trials two of which should occur in different locations, and where the effect is being claimed, be statistically significant ($P < 0.05$) (Anadón, Rosa Martínez-Larrañaga, and Aranzazu Martínez 2006). The trials need to be under farm conditions and have scientific evidence of safe use for the user, consumer, animal, and environment (Anadón, Rosa Martínez-Larrañaga, and Aranzazu Martínez 2006). The health claims by the additive should be related to reduced morbidity and mortality for the target species, and improved feed conversion, performance, and product quality (Anadón, Rosa Martínez-Larrañaga, and Aranzazu Martínez 2006).

Tolerance studies

Tolerance studies come into play with additives that are not on the QPS list and/or are novel (Rychen et al. 2017). These studies are to be carried out to show the efficacy and safety of the additive in question for the target species, particularly in the case of an accidental overdose of the additive during the production of the feed (Anadón, Rosa Martínez-Larrañaga, and Aranzazu Martínez 2006). The clinical, morbidity and mortality, as well as zootechnical parameters; weight gain, feed intake, and feed conversion ratio, need to be carefully monitored and the trial should involve an at least 10-fold the maximum recommended dosage as that proposed by the applicant (Anadón, Rosa Martínez-Larrañaga, and Aranzazu Martínez 2006).

Toxicity, virulence factors, hemolytic, and cytotoxic activity

Those strains that are known to be toxin producers and/or have virulence factors, such as *Bacillus spp.* and *Enterococcus* spp., need to undergo additional tests to show safety (Anadón, Rosa Martínez-Larrañaga, and Aranzazu Martínez 2006). The assessment should look for any presence of mobile genetic elements that can confer virulence and resistance apart from the known toxin and virulence genes. Therefore, the additive should not have any active virulence or toxin genes (Laulund et al. 2017). In that vein, oral and genotoxicity studies should be performed as well.

Antibiotic susceptibility and resistance

A complete antibiotic susceptibility and resistance profile need to be compiled on the potential probiotic additives in question, especially to monitor and determine the ability of the strain to transfer resistance (Anadón, Rosa Martínez-Larrañaga, and Aranzazu Martínez 2006; Laulund et al. 2017) The best test for this evaluation is the *in-vitro* determination of the minimum inhibitory concentration (MIC) against a list of relevant critically and highly important in human and veterinary antibiotics that are of particular importance and can be followed using the methods prescribed in ISO 10932:2010 or Clinical Laboratory Standard Institute (CLSI) (Laulund et al. 2017). The breakpoint values provide an acceptable range for which the additives should fall under (Anadón, Rosa Martínez-Larrañaga, and Aranzazu Martínez 2006; Laulund et al. 2017). Having n MIC value above the breakpoint is not necessarily an automatic dismissal of the additive. Further tests need to be performed whether the resistance is intrinsic or acquired. Intrinsic resistance, depending on the strain and other safety profile, should be acceptable, whereas acquired resistance mainly due to the presence of exogenous resistance genes is problematic as it may be transmitted to other strains and therefore this strain would not be permitted to be used as a feed additive (Anadón, Rosa Martínez-Larrañaga, and Aranzazu Martínez 2006).

Interactions with the gut microbiota

Owing to the large number of studies published on providing alternatives to antibiotics via probiotics, the researchers should test their potential strains for adherence to enterocytes to show the ability of their selected strain to colonize and persist in the mucosa, modulate the microbiota, and exert its health benefit, whether that be a production of bacteriocins or SCFAs or even simply prevent attachment of

pathogens via competitive exclusion. Seeing how these probiotics will act competitively with the microbiota, enterocytes originating from the host organism should be used for in vitro and ex vivo models hence reproducible cell cultures should be available in a commercial capacity to allow for optimized and reproducible techniques for assays (Kaiser et al. 2017).

The effect of the additive on the intestinal microbiota of the target species needs to be monitored and documented (Anadón, Rosa Martínez-Larrañaga, and Aranzazu Martínez 2006). Such studies can be assessed either *ex-vivo* or *in-vivo,* preferably both, by performing gut simulation experiments or by performing target species trials. The goal is to determine whether the addition of the additive has a detrimental impact on the microbiota by either causing an overgrowth or shedding of pathogenic bacteria (Rychen et al. 2018). A limited number of studies exist in the literature that has performed such simulated trials, however, rarely intending to determine the performance of the potential isolated probiotic strain against any common pathogenic avian strains (Kallapura et al. 2015; Card et al. 2017; Crhanova et al. 2019; Oladeinde et al. 2019; Priyodip and Balaji 2019).

The age of the host is the greatest determinator of the chicken gut microbiota (Maki et al. 2019). The intervention must be tested from the day of hatch until commercialization at the time of processing, this allows for determining the ideal time for application of the intervention to achieve the greatest benefit in terms of performance and health. Kumar et al. (2018) performed such a study on chickens from the day of hatch until they gained enough weight to be able to be commercially processed in order to determine the effect of withdrawing antibiotics on the ileal and cecal microbial community, the performance of the birds, host immunity, and prevalence of *Salmonella* and *Campylobacter* in the gut of broilers. They found that at hatch, day 0, no *Salmonella* spp. were found in either the ileum or ceca contents; however, the prevalence of *Salmonella* and *Campylobacter* increased during the early growth stage of the bird while their abundance decreased with age (Kumar et al. 2018). This signifies the importance of applying the intervention early on in the age of the bird, especially if the intention is to help control pathogenic strains from colonizing and proliferating in order to have the maximum impact on the performance and health of the bird (Maki et al. 2019).

Prior to the commercialization of the potential bacteriocinogenic probiotic, thought must be given to all the factors that affect the gut microbiota. One way to determine the effect of different factors on the microbiota is to simulate chicken gut trials. It is not enough to perform in vitro tests on the probiotic strain and then recommend that potential strain as an alternative to antibiotics without having gone a step further and performing the simulated trials. This would also prevent any discrepancies that might arise between in vitro and in vivo testing. Simulated gut trials would also be able to show whether the action of the probiotic would be beneficial or detrimental prior to conducting in vivo trials, thereby potentially preventing the use of chickens altogether while being cost-effective at the same time. The microbiota should be modulated in a positive manner where pathogenic strains are inhibited while bacteriocinogenic strains are increased, as increasing or decreasing specific members of the microbiota can make the difference between weight gain or loss (Maki et al. 2019). It is also important that the probiotic strain can adhere to the mucosa in the presence of commensals; therefore, another recommendation would be to isolate potential probiotic strains from the mucosa of the intestinal tract (Maki et al. 2019). Diet of the chicken is also important to take into consideration when selecting a potential probiotic strain to be tested ex vivo or in vivo as any minor changes in the composition of the feed, such as changing the carbohydrate or protein source or increasing or decreasing carbohydrates or proteins, can have a significant impact on the gut microbiota (Kumar et al. 2018).

Interactions with host cell-lines

To date, there is a severe lack of commercially available avian gastrointestinal epithelial cell lines, unlike the abundance of intestinal cell lines available for other mammalian animals (Rath et al. 2018). As an alternative, the LMH (ATCC CRL-2117) cell lines which are chemically induced hepatic cellular carcinoma cells or DIV-1 which are moderately differentiated intestinal epithelial cells are used as seen in these studies (Van Immerseel 2003; Konkel et al. 2007; Larson et al. 2008; Flanagan et al. 2009; Spivey, Dunn-Horrocks, and Duong 2014; Li et al. 2019). A few studies have drafted protocols on isolating chicken enterocytes to fill the gap, but these are not commercially available, add time and money to an already expensive venture, and have utilized different techniques (Velge 2002; Dimier-Poisson, Bout, and Quéré 2004; Yuan et al. 2015; Ding et al. 2017; Kaiser et al. 2017; Rath et al. 2018). It appears that obtaining continuous cell lines from chicken tissues has always presented difficulties (Velge 2002). In the past, there were some commercial avian intestinal cell lines available; however, they either were confirmed to originate from a specie other than chickens, or they lacked some characteristics of polarized epithelial cells (Kaiser et al. 2017). The relatively short in vitro survival time and considerable cell death shortly after plating might explain this reluctance to produce any new commercial intestinal cell lines (Velge 2002). This also might explain why a limited number of studies perform adhesion assays, and even fewer use avian cell lines. While human tumour-derived cells such as Caco-2 cells have been used in studies involving probiotics and their interaction with epithelial cells (Spivey, Dunn-Horrocks, and Duong 2014; Kaiser et al. 2017), carcinogenesis is an altered state of cells that may exhibit altered genetics which in turn may alter their function and hence skew the results. Importantly, if the potential probiotic strains are to be commercialized in order for their use in the poultry industry, in vivo testing of these strains on chicken enterocytes should be considered. Finally, hemolytic and cytotoxicity assays should also be performed to determine the ability of the additive to cause harmful effects on the cells due to the ability of the bacteriocinogenic strains to colonize the lining of the GIT. This

would be particularly important in the case of additive preparations for animal feed.

Requirements for the commercialization of novel food additives

Novelty implies that the additive has not been widely used in the past and, as such, does not have a history of safe use. Organisms that have been genetically modified fit into these criteria as well (Laulund et al. 2017). The EU's regulation regarding novel foods can be found in the regulation EU 2015/2283, which states that "The novel foods and food ingredients concerned by this regulation are those who are not yet currently used for human consumption" (Laulund et al. 2017). These additives must undergo a pre-market evaluation, a risk assessment by the European Food Safety Authority (EFSA), and risk management by the EU Commission before its commercialization (Laulund et al. 2017). Similarly, in Canada, novel ingredients not listed in Schedule IV or V of the Feeds Regulations by the CFIA must undergo a complete safety and efficacy assessment as part of the pre-market assessment, which involves assessment of new ingredients and product registration (Canadian Food Inspection Agency 2019). Additionally, any ingredients listed in Schedule IV or V that are different in composition, structure, nutritional quality or physiological effects, its purpose, its manufacturing process, its safety, its metabolized form in the target species is required to undergo a new safety and efficacy assessment (Canadian Food Inspection Agency 2019). In the US, a pre-market approval with a complete safety assessment is required by the CVM.

Conclusion

With the continued rise in the human population, there is going to be an increase in the demand for safe, nutritious, and cheap animal protein sources. Bacteriocins have a long history of safe use in food, and while their application has been constrained to food preservation for many years, bacteriocins are slowly gaining the recognition needed due to their strong and ideal characteristics, particularly in terms of pathogen control. Furthermore, application of the potential physiological properties of these molecules is only at its beginning, mainly due to difficulties associated with their current cost-prohibitive large-scale production, with the use of bacteriocinogenic probiotics is considered as a promising alternative. These microbes would also have the advantage of providing additional benefits to the host, such as improving host phenotype due to the ability to colonize the gut mucosa due to competitive exclusion. This competitive advantage would then allow them to proliferate, prevent pathogenic strains colonization, as well as help in the elimination of existing colonized pathogens by the release of bacteriocins as well as SCFAs. Many details are still needed to be worked out in terms of the ideal route of delivery, dosage, and manufacturing processes to not only maintain efficiency but effectiveness as well. We have laid out the mandatory criteria in order to commercialize a bacteriocinogenic probiotic product. Despite the several hurdles that must be overcome for the exploitation of bacteriocinogenic bacteria in livestock and food systems, the innovations, developments, and regulations discussed in this review offer a taste of future trends in animal feed applications of these promising microbes.

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