Isolation and Characterization of Bacterial Probiotic Candidates from Chicken Gut with Bacteriocinogenic Properties

Ayesha Lone

Thesis submitted to the University of Ottawa in partial
Fulfillment of the requirements for the
Master of Science (MSc)

Interdisciplinary Health Sciences
Faculty of Health Sciences
University of Ottawa

Abstract

Exposure to chicken and other poultry products has been identified as both a common source of campylobacteriosis and salmonellosis outbreaks and as a risk factor for sporadic infection with these pathogens. With the recent ban on the use of antibiotics as growth promoters and for prophylaxis, producers will have to change the way the industry raises broilers by trying to mitigate the spread of poultry pathogens while maintaining the productivity of the past. The main objective of this research project was to isolate and characterize bacterial strains from broiler chicken gastrointestinal tract with inhibitory activity against *Salmonella* and *Clostridium perfringens*. Several isolates from the caecal mucosa have been identified and their activity characterized. Of 290 active colonies pre-selected using a double-layer technique, a total of 31 colonies were able to produce a supernatant inhibiting *Salmonella* Abony and *Clostridium perfringens* and were identified as *Ligilactobacillus salivarius*, *Ligilactobacillus agilis*, *Lactobacillus johnsonii*, *Lactobacillus. kitasatonis*, *Lactobacillus* sp., and a new uncultured bacterium. Five most potent strains were further characterized for their probiotic potential (i.e., sensitivity to antibiotics and tolerance to gastrointestinal physicochemical conditions). Importantly, all five strains harbor bacteriocin genes, as evidenced by performing whole-genome sequencing.

Keywords: probiotics, poultry, *Salmonella*, *Clostridium perfringens*, antimicrobial activity, bacteriocins

Résumé

L'exposition aux poulets et autres produits de volaille a été identifiée à la fois comme une source commune de campylobactériose et de salmonellose et comme un facteur de risque d'infection sporadique par ces agents pathogènes. Avec l'interdiction récente de l'utilisation d'antibiotiques comme facteurs de croissance et à titre prophylactique, les producteurs devront modifier la façon dont l'industrie élève les poulets de chair en essayant d'atténuer la propagation des agents pathogènes des volailles tout en maintenant la productivité du passé. L'objectif principal de ce projet de recherche était d'isoler et de caractériser les souches bactériennes du tractus gastro-intestinal des poulets de chair ayant une activité inhibitrice contre *Salmonella* et *Clostridium* perfringens. Plusieurs isolats de la muqueuse caecale ont été identifiés et leur activité a été caractérisée. Sur 117 colonies actives présélectionnées à l'aide d'une technique à double couche, 31 colonies au total ont été capables de produire un surnageant inhibant *Salmonella* Abony et *Clostridium perfringens* et ont été identifiées comme étant *Ligilactobacillus salivarius*, *Ligilactobacillus agilis*, *Lactobacillus johnsonii*, *Lactobacillus kitasatonis*, *Lactobacillus* sp., et une nouvelle bactérie non cultivée. Cinq souches les plus actives ont été caractérisées pour leur potentiel probiotique (tel que leur sensibilité aux antibiotiques et leur tolérance aux conditions physico-chimiques gastro-intestinales). Il est important de noter que les cinq souches abritent des gènes de bactériocine, comme le montre le séquençage du génome entier.

Mots-clés: probiotiques, poulet, *Salmonella*, *Clostridium perfringens*, activité antimicrobienne, bactériocines

Acknowledgements

I would like to express my deep gratitude to my research supervisor, Dr. Riadh Hammami, who gave me the opportunity to do valuable and meaningful research. He has always been incredibly patient and generous with his time and has provided me with opportunities to advance in my field through learning new skills and helping develop and improve existing ones.

I would also like to thank my advisory committee, Drs. Frederique Tesson, Maxwell Hincke, and Krista Power, for their encouragement, suggestions, and insightful comments.

This project would not be possible without financial support from OMFARA. I am particularly grateful to the regional veterinarian, Richard D Smith, for providing the necessary paperwork to work with the chicken gastrointestinal tracts, and Angela from Berube Poultry, for providing the tracts.

A warm thanks to my friends and research colleagues, Yasmina Ait Chait, Rojaalsadat Mousavi, and Walid Mottawea, for their valuable assistance and constant encouraging words. Special thanks to Ray and Bond and gratitude to all the friends and family who supported me throughout this journey, directly and indirectly.

Lastly, a heartfelt thank you to my parents for their love, words of encouragement, wisdom, and unwavering support. Thank you for instilling in me a strong work ethic but, above all, love and thirst for knowledge.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AMR	Antimicrobial Resistance
ARG	Antibiotic Resistance Genes
BIOHAZ	Panel on Biological Hazard
CDC	Centers for Disease Control
CFIA	Canadian Food Inspection Agency
CFS	Cell free Supernatant
CLSI	Clinical Laboratory Standard Institute
CVM	Center for Veterinary Medicine
EFSA	European Food Safety Agency
EU	European Union
FAO	Food and Agriculture Organization
FCR	Feed Conversion Ratio
FDA	Food and Drug Administration
FFDCA	Federal Food, Drug, and Cosmetic Act
FI	Feed Intake
GIT	Gastrointestinal Tract
GRAS	Generally Recognized As Safe
IDF	International Dairy Federation
LAB	Lactic Acid Bacteria
MIC	Minimum Inhibitory Concentration
MRS	de Man, Rogosa, and Sharpe
NE	Necrotic Enteritis

OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
QPS	Qualified Presumption of Safety
rRNA	Ribosomal Ribonucleic Acid
SCFAs	Short Chain Fatty Acids
SIJ	Simulated Intestinal Juice
SGJ	Simulated Gastric Juice
TSBYE	Tryptic Soy Broth Yeast Extract
UK	United Kingdom
USA	United States of America
VMPs	Viable Microbial Products
WGS	Whole Genome Sequencing
WHO	World Health Organization
WPA	Australian Working Party on Antibiotics

Introduction

The poultry gastrointestinal tract (GIT) consists of the crop, gizzard, duodenum, ileum, and ceca (Micciche et al., 2018c). The GIT is shorter than that of other food-producing animals and has different food retention times and pH variations throughout the intestinal tract (Micciche et al., 2018; Pan & Yu, 2014). The entire tract's average transit time is approximately 3.5 hours (Pan & Yu, 2014). Together, this unique environment has allowed various microorganisms to adapt to these conditions and thus have formed the poultry microbiome, which contains microorganisms that can adhere to the mucosal layer and proliferate at a fast rate (Pan & Yu, 2014).

The poultry microbiome is heavily populated by Firmicutes, Bacteroidetes, and Proteobacteria, accounting for more than 90% of the intestinal bacterial phyla, with *Lactobacillus*, *Bifidobacterium* spp, *Enterococcus faecium*, and *Pediococcus* spp. being the most abundant in the small intestine, with the highest concentration of anaerobic bacteria being found in the ceca (Micciche et al., 2018; Pan & Yu, 2013). The cecum is also the colonizing site of pathogenic bacteria such as *Salmonella* spp., *Escherichia coli*, *Clostridia* spp. and *Campylobacter* spp. (Adhikari & Kwon, 2017; Oakley et al., 2014). The poultry microbiome is globally considered to be a major source of foodborne infections. In Canada, 1 in 8 Canadians are affected by a foodborne illness that results in 11,600 hospitalizations and 238 deaths (Thomas et al., 2013).

Salmonella and *Clostridium perfringens* are two leading pathogens that cause foodborne illnesses and are linked explicitly to poultry products (Hofacre et al., 2018; Liu et al., 2018). The Centers for Disease Control (CDC) estimates that 1 million cases of *C. perfringens* and 1.35 million infections of *Salmonella* occur per year (CDC, 2018a, 2020). In the European Union (EU), one in four infections in humans caused by *Salmonella* show resistance to three or more antimicrobials routinely used in human and animal medicine (Food et al., 2018). These pathogens cause a high mortality rate with their outbreaks and significant economic losses to the poultry industry.

Salmonellosis and Necrotic Enteritis (NE), a disease caused by *C. perfringens* in chickens, usually occurs due to a damaged intestinal tract and are indirectly related to each other (Hofacre et al., 2018). The intestinal tract can become damaged either through another disease, coccidiosis caused by *Eimeria* species, or through the use of live vaccines (Hofacre et al., 2018). Intestinal damage causes increased mucus production, which ultimately results in increased feed conversion ratio (FCR), reduced weight gain, and increased growth and proliferation of *C. perfringens*, which is usually observed for sub-clinical NE disease (Hofacre et al., 2018). The same situation applies when live vaccines are administered. As *C. perfringens* resides in the ceca, a proliferation would mean increased toxin production, which can be transported to the small intestine via retro-peristalsis, a normal phenomenon, resulting in further intestinal damage (Hofacre et al., 2018). Coccidiosis caused by *Eimeria tenella* also results in higher levels of *Salmonella* Typhimurium in the spleen and liver of chickens due to its spread from the ceca to the damaged intestinal tract. Therefore, the control of *Eimeria* species will result in the control not only of *C. perfringens* but also of *Salmonella* and vice versa, as *C. perfringens* cannot proliferate unless a damaged intestinal tract exists (Hofacre et al., 2018).

Traditionally, poultry producers used antimicrobials to control these foodborne pathogens. Antimicrobials were used therapeutically, prophylactically, or as growth promoters in animals at sub-therapeutic levels on an almost daily basis in greater numbers (Marshall & Levy, 2011a; Pan & Yu, 2013a). Usually, the classes of antimicrobials used in animals were the same as those used in human medicine due to the limited number of therapeutically useful antibiotics (Prescott, 2008).

Approximately 13.6 million kilograms of medically important antimicrobials approved for food-producing animals were sold and distributed in the US in 2016 (Cvm, 2017).

The presence of antibiotics in the feed, such as avoparcin and bacitracin, also has a significant influence on the microbiome, causing not only dysbiosis but increasing the likelihood of commensal bacteria from acquiring resistance genes via horizontal gene transfer and becoming pathogenic with increased virulence (Card et al., 2017a; Oakley et al., 2014a). Considering 70% of the antibiotics added in feed or drinking water are excreted in an un-metabolized form, animal waste contributes to adding resistant genes into the environment (Suresh et al., 2018). With some of the waste being used as litter in the poultry industry and for multiple cycles, chickens can easily acquire these resistance genes via horizontal gene transfer contributing to AR pathogens internally and externally (Pan & Yu, 2013a). Use of non-therapeutic antimicrobials in food animals inevitably results in the exposure of farmers, farm workers, rural communities, and the general public to resistant pathogens, as well as causes contamination of air, water, and soil near food animal production sites (Silbergeld et al., 2008). Importantly, the increased use of antibiotics results in the expansion of reservoirs of resistance because these genes can be transferred widely among microbial communities (Silbergeld et al., 2008).

It is no surprise then to learn that extensive literature exists depicting the high prevalence of antibiotic resistance in pathogenic bacteria isolated from retail meat and poultry products, many of which show multi-drug resistance (Qiao et al., 2017; Trung et al., 2017; Zhu et al., 2017). The capacity of a microorganism to resist the inhibitory or killing activity of an antimicrobial to which it was once susceptible is known as antimicrobial resistance (AMR) (Verraes et al., 2013). AMR is currently considered to be the biggest threat facing public health. It has been estimated that by 2050 approximately 10 million deaths a year will occur as a result of AMR and will cost the world economy $100 trillion annually (Eloit et al., 2016; O'Neill, 2014). The increased use of antimicrobials has resulted in the inevitable, rapid acclimatization and development of resistance in humans and food-producing animals. Production of new antimicrobials has been limited, and only eight innovative products are currently in development. Consequently, researchers have been looking for effective alternatives to combat resistance and mitigate the spread of antibiotic-resistant bacteria, particularly for their use in food animals such as probiotics.

Probiotics have been defined as live microorganisms that provide health benefits to the host (Bajagai et al., 2016). Probiotics have a good safety record and are currently being used in the dairy industry as health supplements and direct feed microbials in chickens' feeds. *Lactobacillus*, *Bifidobacterium*, *Enterococcus* spp, *Leuconostoc* spp, and *Saccharomyces spp* are commonly used as probiotics (M. L. Wan et al., 2018). As they have been around for a long time and found in many food products, they have gained public acceptance as being natural and environmentally safe. FAO and WHO recommend that a probiotic be able to survive the passage, adhere, and multiply through the GIT, be a gram-positive organism, show measurable health benefits, and have a defined dosage duration (Patterson, 2008; M. L. Wan et al., 2018). Probiotics have been shown to modulate the immune system, increase proliferation of beneficial commensal bacteria, nutrient absorption, feed conversion efficiency, and body weight (Suresh et al., 2018). For some probiotics, such as *Lactobacilli*, these beneficial properties are due to the production of bacteriocins and short-chain fatty acids.

Bacteriocins are small ribosomally synthesized peptides that are multi-functional and show a narrow spectrum of antimicrobial activity at certain concentrations (Chikindas et al., 2018a; Flint & Garner, 2009). Bacteriocins work by inserting on the cell membrane of antagonistic bacteria and creating a pore in the cell membrane resulting in cell destruction (Flint & Garner, 2009).

Bacteriocins have traditionally been used as food preservatives, with Nisin being the only food-grade bacteriocin being granted GRAS (generally recognized as safe) status for certain applications (Chikindas et al., 2018a).

Short-chain fatty acids (SCFAs) are organic acids, such as lactic acid, propionate, butyrate, and acetate, produced by some commensal bacteria inhibitory to certain pathogenic bacteria (Pan & Yu, 2013a). SCFAs are also produced by certain probiotic strains, or they can be added to chicken feed, drinking water, and other matrices as they have been shown to improve growth, feed quality and modulate disease response (Santin et al., 2017; Suresh et al., 2018). SCFA-producing bacteria release acids due to the fermentation of carbohydrates present in the feed, which lowers the pH of the GIT (Pan & Yu, 2013a). This low pH has an antibacterial effect on the antagonistic bacteria.

The poultry industry is expected to be the main agricultural industry to continuously grow in the upcoming years, between 2005 and 2050, with a 121% increased demand for meat and 65% for eggs owing to increased consumer demand (Mottet & Tempio, 2017a). With the recent bans on antibiotics use, the industry is now facing the challenge of maintaining the same productivity and profits while producing safe products. Unfortunately, antimicrobial withdrawal has a major impact on gut health in intensively reared broiler chickens, as witnessed in Europe following the ban of antibiotic growth promoters in 1999 (Hofacre et al., 2018). Therefore, a healthy intestinal tract and a stable microbiome must be maintained in broilers, which will confer the advantage to ensure a low FCR, increased weight gain, and a higher meat yield (Gaucher et al., 2017). This project investigates bacteriocinogenic bacteria as an integrated nutritional approach for the positive modulation of the intestinal microbiota while reducing the incidence of pathogens in chicken

Chapter 1

BACTERIOCINOGENIC PROBIOTICS AS AN INTEGRATED ALTERNATIVE TO ANTIBIOTICS IN CHICKEN PRODUCTION - WHY AND HOW?

Ayesha Lone[1], Walid Mottawea[1, 2], Youcef Mehdi[3], Riadh Hammami[1, *]

[1] N*u*GUT Research Platform, School of Nutrition Sciences, Faculty of Health Sciences, University of Ottawa, Ottawa, Ontario, Canada

[2] Department of Microbiology and Immunology, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.

[3] Faculté des sciences de l'agriculture et de l'alimentation, Université Laval, Québec, Canada

* Corresponding author. E-mail address: riadh.hammami@uottawa.ca

Paper submitted for publication in *Critical Reviews in Food Science and Nutrition*

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: bacteriocin, livestock, poultry, alternative to antibiotics, regulations

1. Introduction

The poultry industry plays a significant economic role in both developing and developed countries and is the fastest-growing agricultural sub-sector (Alali & Hoface, 2016; Mottet & Tempio, 2017b). Poultry meat and eggs are the most common animal source of protein consumed, and the second most-consumed meat globally (Alali & Hoface, 2016; Mottet & Tempio, 2017b). 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 & Tempio, 2017b). 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 & 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 & Tempio, 2017b). 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 *Campylobacte*r infections in humans (Alali & 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.

1. Antibiotics in Poultry Industry

1.1. 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 & Levy, 2011b; Pan & Yu, 2013b). 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 et al., 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 et al., 2017). Therefore, poultry and its products can be a source of introduction or dissemination of antibiotic-resistant pathogens (Agyare et al., 2019; Holmes et al., 2016). 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, 2015a). 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 (CDC, 2018b). In addition, the gut microbiota of poultry and other farm animals act as a large reservoir for resistance genes (Y. Wang et al., 2019).

1.2. 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 & Yu, 2013a). 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., 2014b). Sex, age, feed additive, antibiotic, geography, litter type, and host genotype are just some of the factors that can influence the chicken's microbiota (J. Li et al., 2017; Micciche et al., 2018a; Pan & Yu, 2013b). 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., 2014b). The microbiota eubiosis can be disrupted by antibiotics that may be used therapeutically, prophylactically, or for growth promotion, favouring certain bacterial classes over others (J. 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 (J. 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 caecal microbiota by causing a reduction in the existing dominant species such as *Lactobacillus* (Crisol-Martínez et al., 2017).

2. Global Regulations on the Ban of Antibiotics for Growth Promotion in Animals

Antimicrobial consumption is expected to rise by almost 70% by 2030 (Mottet & Tempio, 2017b). 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 et al., 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 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 Organisation for Animal Health (OIE) and the Food and Agriculture Organisation 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).

2.1. 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 (Renée Johnson, 2009). 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 (Ryan 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 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.

2.2. 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 et al., 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 endeavour 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, 2018b) 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 (Government of Canada, 2018b; The Chicken Farmers, 2016). 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, 2018a).

2.3. 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, J., 2019; Wu, Z., 2019). This is ambitious as it was estimated that in 2013, 84,240 tons of antibiotics had been used in animal production in China (H. 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, J., 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 (Ralte, 2018; Sri Lanka, 2017).

2.4. 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).

2.5. 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, J., 2019).

3. Bacteriocins as Promising Alternatives for the Poultry Industry

3.1. 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., 2018b). The anti-infective potential of bacteriocins for inhibiting pathogens has been shown in various food matrices, including cheese, meat and vegetables (Hammami et al., 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 these 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., 2010a). 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 (Cavera et al., 2015; Damian Józefiak & Sip, 2013). These bacteriocins contain lanthionine in their structure, have a molecular mass of under 5kDa, and are thermostable, membrane-active peptides (Damian Józefiak & 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 (Cavera et al., 2015; Damian Józefiak & Sip, 2013). 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 (Damian Józefiak & Sip, 2013). Class II bacteriocins are further sub-classified; IIa-pediocin like bacteriocins, IIb- dipeptide bacteriocins, IIc- sec-dependent bacteriocins, and IId-bacteriocins (Jozefiak D, Sip A, 2013). Class III bacteriocins are non-membrane active peptides that have a molecular mass of higher than 30kDa and are thermolabile (Jozefiak D, Sip A, 2013).

Lastly, class IV bacteriocins are those that form protein-lipid or protein-carbohydrate complexes (Jozefiak D, Sip A, 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 16s 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 (Cavera et al., 2015; Chugunov et al., 2013). 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).

3.2. 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 (Damian

Józefiak & 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 signalling 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., 2018b). 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; M. L. L. Y. Wan et al., 2018). 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 (M. L. L. Y. Wan et al., 2018). The inhibition of pathogens in the GIT is usually through this mechanism (M. L. L. Y. Wan et al., 2018). 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).

2.6.1 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 (Damian Józefiak & 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 (Damian Józefiak & 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 favourable 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.

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 (Damian Józefiak & 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.

2.6.2 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 to gut contents (E. A. Svetoch & Stern, 2010). (E. A. Svetoch & 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., 2017a). 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. reuter*i L22, was found to be able to significantly reduce the growth of *Salmonell*a 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 (Dexian 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 (J. Y. Kim et al., 2015).

4. Food Safety Regulations and Policies for Commercial Use of Bacteriocins

4.1. 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 genetic elements, antibiotic production and resistance (Anadón et al., 2006). The strain or bacteriocins' stability and any incompatibilities with other feed ingredients should also be stated (Anadón et al., 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.

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

4.2.1. 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., 2018a). 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 et al., 2006). The QPS can be considered to be the equivalent of the Generally Recognized as Safe (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).

4.2.2. Canada

4.2.2.1 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 labelled 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, colouring, foaming or flavouring 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 flavours, 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).

4.2.2.2 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).

4.2.3. 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 labelling and regulations etc. (Fields, 1996; Food and Drug Administration, 2019).

4.3. Criteria for selection of bacteriocinogenic bacteria as feed additives

4.3.1. 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 et al., 2006;

Rychen et al., 2018a). 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., 2018a). Prior to using WGS, a complete taxonomic characterization must already be performed (Laulund et al., 2017).

4.3.2. 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 et al., 2006). The trials need to be under farm conditions and have scientific evidence of safe use for the user, consumer, animal, and environment (Anadón et al., 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 et al., 2006).

4.3.3. 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 et al., 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 et al., 2006).

4.3.4. 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 et al., 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.

4.3.5. 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 et al., 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 et al., 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 et al., 2006).

4.3.6. 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 et al., 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., 2018b). 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 (Card et al., 2017b; Crhanova et al., 2019; Kallapura et al., 2015; Oladeinde et al., 2019; Priyodip & 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 caecal 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).

4.3.7. 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 (Flanagan et al., 2009; Konkel et al., 2007; Larson et al., 2008; Q. Li et al., 2019; Spivey et al., 2014; Van Immerseel, 2003). 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 (Dimier-Poisson et al., 2004; Ding et al., 2017; Kaiser et al., 2017; Rath et al., 2018; Velge, 2002; Yuan et al., 2015). 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 (Kaiser et al., 2017; Spivey et al., 2014), 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.

5. 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.

6. 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.

Acknowledgements

This study was supported by a grant from the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) [No. OAF-2019-100380].

Disclosures

The authors declare no financial conflicts of interest.

Table 1.1. Antibiotic categories, class, and route of administration in broiler chickens adapted from (Government of Canada, 2018a).

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 Penicillin-streptomycin
	Quinolones (except fluoroquinolones)		
	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

Table 1.2. 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	(D Jozefiak et al., 2011; D. Józefiak et al., 2012; Damian Jozefiak et al., 2011)
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	(Damian 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*		(Edward A. Svetoch et al., 2011)
SMXD51	*Lactobacillus salivarius* SMXD51	Chicken ceca	*C. jejuni, C. coli*		(S. Messaoudi et al., 2013a; Soumaya Messaoudi et al., 2011)
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	(H. T. Wang et al., 2011, 2013)

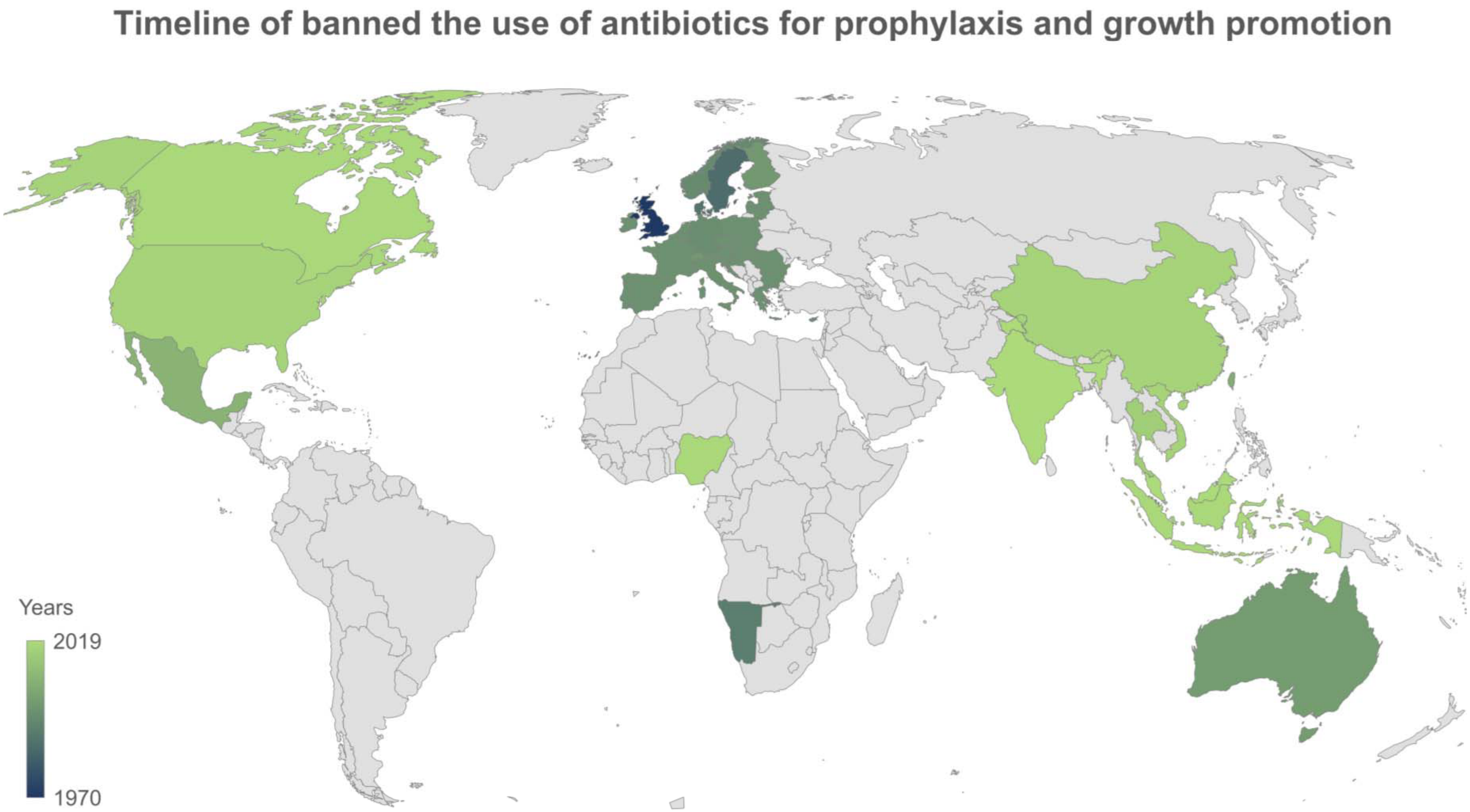

Figure 1.1. Timeline of countries that have banned the use of antibiotics for prophylaxis and growth promotion.

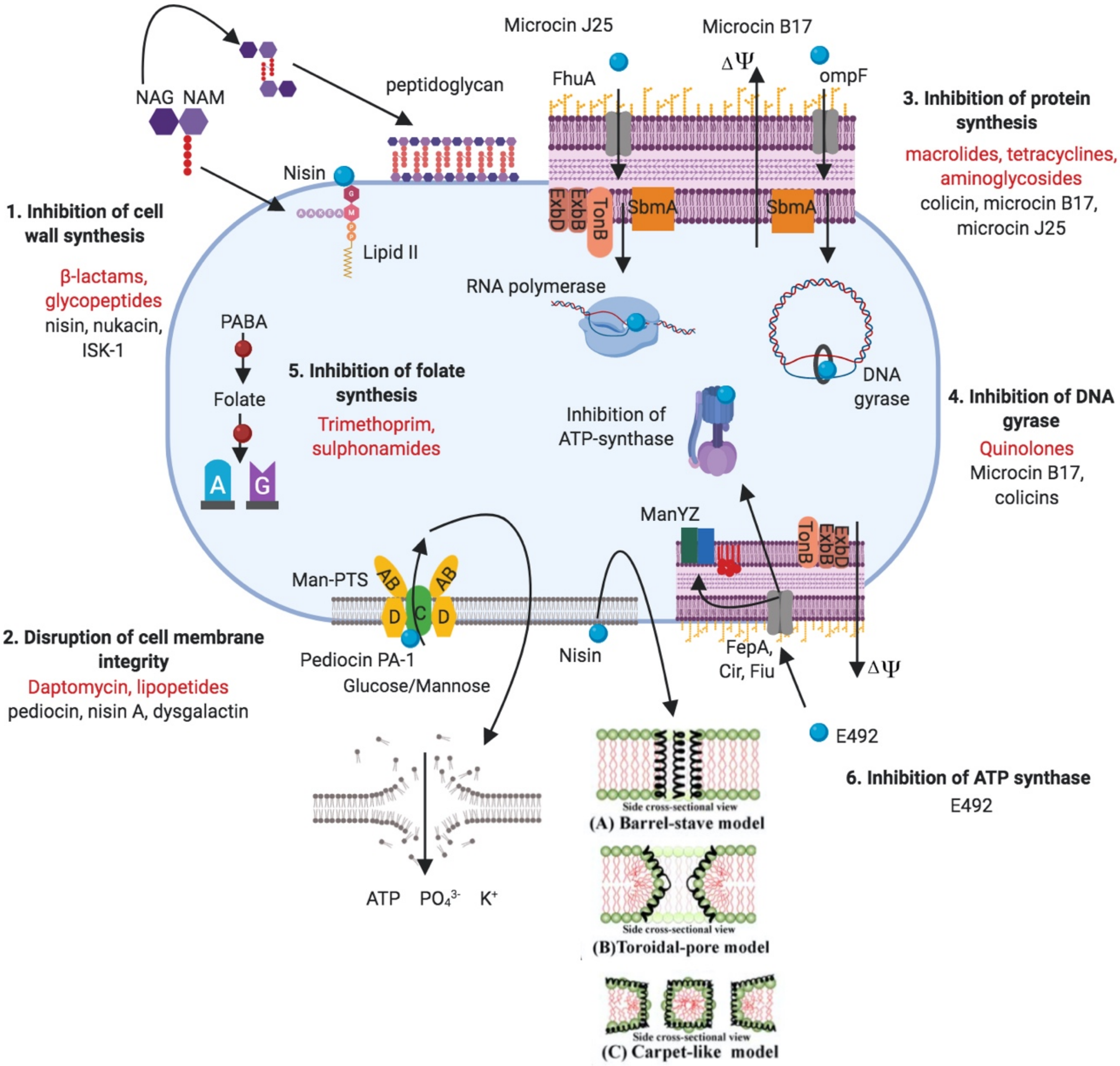

Figure 1.2. Overview of the similarity in targets between bacteriocins and antibiotics. Created with Biorender.com.

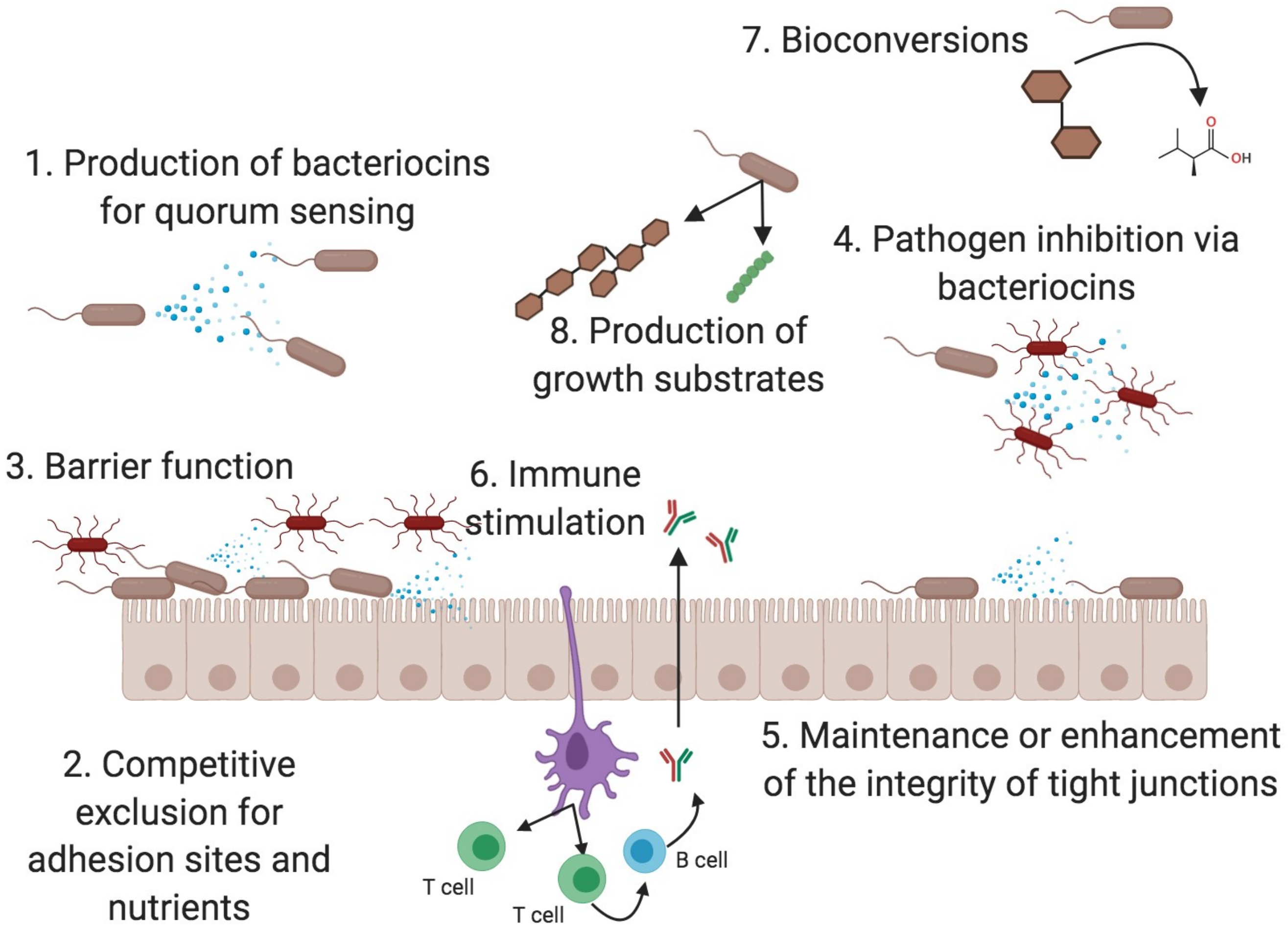

Figure 1.3. Overview of the mechanisms of interaction of bacteriocinogenic strains in the intestinal tract. Adapted from (O'Toole & Cooney, 2008).

Created with Biorender.com.

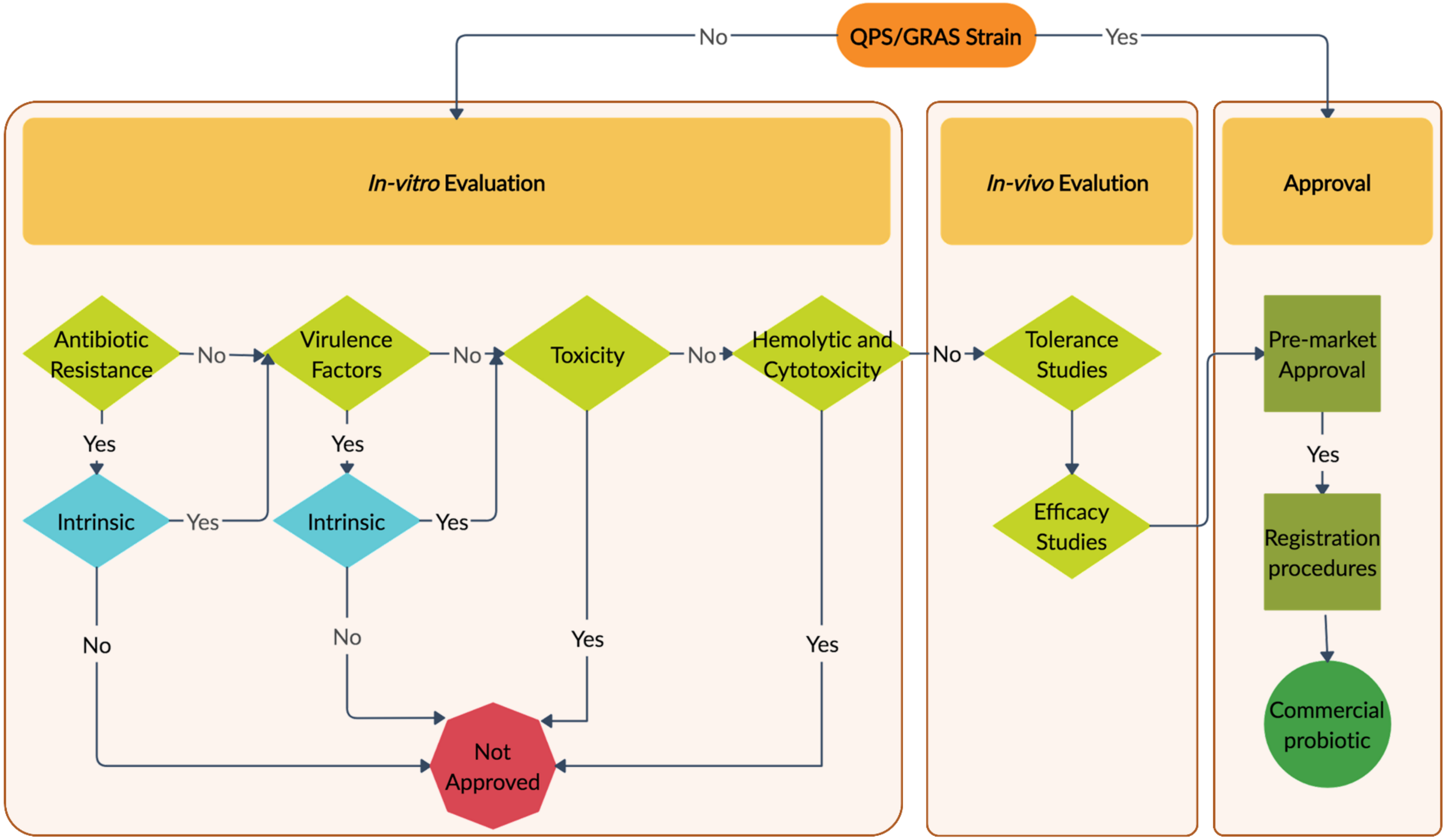

Figure 1.4. Illustration of the current steps required for the commercialization of bacteriocinogenic probiotics.

Hypothesis and objectives

With increased consumer demand for natural, organic, and antibiotic- free meat, coupled with recent policies in place to ban the use to antibiotics for non-therapeutic use, the food industry is under pressure and running out of time to find a viable alternative to antibiotics that is low cost, allows maximization of host feed utilization, promotes weight gain, and is able to act as a prophylactic. This is where bacteriocin-producing probiotics come in. While many studies exist that show beneficial effects of such probiotics in chickens, *in vitro*, *in vivo,* or both, the results have been inconsistent (Ben Lagha et al., 2017b). No study has yet been carried out that exclusively isolates endogenous bacteriocin-producing probiotics from the intestinal mucosa of chicken gastrointestinal tract and then tests its efficacy against both *Salmonella* (Gram negative) and *Clostridium perfringens* (Gram positive). Additionally, it is recommended that probiotics that are to be used in the poultry industry be a normal inhabitant of the gut, be able to adhere to the intestinal epithelium as well as be able to compete with the commensals for colonization sites, be able to withstand the harsh gut environment, such as tolerance to acidic pH and bile salts, be able to exert beneficial effects in the host, and lastly are able to maintain viability after industrial and storage processes and conditions (Kabir, 2009). Isolating potential probiotic candidates from the mucosa of the intestinal tract rather than the gut contents fulfills the first three criteria mentioned above, as those commensals would already have the ability to attach, adhere, proliferate, and hence colonize the intestinal tract. Therefore, our main aim is to isolate novel probiotic candidates from the mucosa of broiler chicken gastrointestinal tract with the potential to inhibit *Salmonella* (Gram negative) and *Clostridium perfringens* (Gram positive).

Our specific aims are:

Aim 1. To screen and characterise the antibacterial activity of mucosal-associated intestinal strains against *Salmonella* and *Clostridium perfringens*

Aim 2. Identification and characterisation of the probiotic potential of newly isolated intestinal bacteria

We hypothesize that our bacteriocin-producing strains isolated ceca mucosa will be able to tolerate acidic pH and bile salts and will be capable of inhibiting both *Salmonella* and *Clostridium perfringens.*

Chapter 2

Dual inhibition of *Salmonella* and *Clostridium perfringens* by new probiotic candidates isolated from chicken intestinal mucosa

Ayesha Lone[1], Yasmina Ait Chait[1], Riadh Hammami[1, *]

[1] N*u*GUT Research Platform, School of Nutrition Sciences, Faculty of Health Sciences, University of Ottawa, Ottawa, Ontario, Canada

* Corresponding author. E-mail address: riadh.hammami@uottawa.ca

Abstract

The poultry industry is the fastest-growing agricultural sector. With poultry meat being economical and in high demand, the end product's safety is of importance. Globally, governments are coming together to ban the use of antibiotics as prophylaxis and for growth promotion in poultry. *Salmonella* and *Clostridium perfringens* are two leading pathogens that cause foodborne illnesses and are linked explicitly to poultry products, with numerous outbreaks occurring every year. A substitute for antibiotics is required by the industry to maintain the same productivity level and hence profits. We aimed to isolate and identify potential probiotic strains from the ceca mucosa of the chicken intestinal tract with bacteriocinogenic properties. We were able to isolate multiple and diverse strains, including a new uncultured bacterium, with inhibitory activity against *Salmonella* Typhimurium ATCC 14028, *Salmonella* Abony ATCC BAA-2162, *Salmonella* Choleraesuis ATCC 10708, *Clostridium perfringens* ATCC 13124, and *E. coli* ATCC 25922. The five most potent strains were further characterized for their probiotic potential (i.e., sensitivity to antibiotics and tolerance to gastrointestinal physicochemical conditions).

Keywords: Poultry, Lactobacilli, Probiotic candidates, antimicrobial activity, *Salmonella*, *Clostridium perfringens*

1. Introduction

Salmonella is one of the leading causes of foodborne diseases worldwide, followed closely by *Clostridium perfringens* (Hofacre et al., 2018; Liu et al., 2018). In the European Union (EU), one in four infections in humans caused by *Salmonella* show resistance to three or more antimicrobials that are routinely used in human and animal medicine ("European Union Summary Report on Antimicrobial Resistance in Zoonotic and Indicator Bacteria from Humans, Animals and Food 2016 Published," 2018; Food et al., 2018). In the US, 5% of *Salmonella* tested was found to be resistant to five or more types of antibiotics in 2011, and by the end of 2018, the number of *Salmonella* outbreaks in the US was sitting at fourteen, five were linked to chickens, raw chickens, and eggs, which was twice as many as seen in 2015 and 2016 (CDC, 2018b; Center for Disease Control and Prevention, 2015b). As for *C. perfringens*, the CDC estimates that it causes 1 million cases of foodborne illnesses per year, with substantial economic loss to the poultry industry due to the high rate of poultry mortality (CDC, 2018a; Immerseel et al., 2004).

Exposure to chicken and other poultry products has been identified as a common source of campylobacteriosis and salmonellosis outbreaks and as a risk factor for sporadic infection with this pathogen (Oakley et al., 2014a). With the recent ban on the use of antibiotics as growth promoters and for prophylaxis (Food and Drug Administration, 2018a; Government of Canada, 2018c), producers will have to change the way the industry raises broilers by trying to mitigate the spread of poultry pathogens while maintaining the productivity of the past. Proposed alternatives to antibiotic use in poultry feeds include phytogenic feed additives, feed acidifiers, antimicrobial peptides, bacteriophages, antibodies, prebiotics, and probiotics (Redondo et al., 2014; Suresh et al., 2018). Probiotics are promising alternative growth promoters, and evidence of their beneficial effects is accumulating in poultry production (Angelakis, 2017). The mode of action of probiotic feed additives in poultry is mainly based on four principles, (i) maintaining normal intestinal microbiota by competitive exclusion and antagonism (Kizerwetter-Swida & Binek, 2009), (ii) altering metabolism by increasing digestive enzyme activity, and decreasing bacterial enzyme activity and ammonia production (Jin et al., 2000), (iii) improving feed consumption and digestion (Awad et al., 2006), and (iv) stimulating the immune system (X. Yang et al., 2014). Furthermore, they exert indirect antimicrobial effects against pathogenic bacteria, which minimize the possibility of developing antimicrobial resistance among the bird commensals, while enhancing the proliferation of beneficial microbes (Suresh et al., 2018). Particularly, bacteriocins and their producing probiotics, such as *Enterococcus* and some *E. coli* spp., have acquired great attention as natural antibiotic alternatives in the food industry (Hanchi et al., 2018; Lauková et al., 2015; X. Yang et al., 2014). However, the prevalence of antimicrobial resistance genes (ARGs) and virulence factors among the investigated strains hinder their application in food industry (Hanchi et al., 2018). While many studies exist that show beneficial effects of such probiotics in chickens, *in vitro*, *in vivo,* or both, the results have been inconsistent (Ben Lagha et al., 2017b). No study has yet been carried out that exclusively isolates endogenous bacteriocin-producing probiotics from the intestinal mucosa of chicken gastrointestinal tract active against both *Salmonella* and *Clostridium perfringens*. In this study, we aimed to isolate potential probiotic candidates from the ceca mucosa of broiler chicken gastrointestinal tract that have the ability to reduce the growth of common poultry pathogens *Salmonella* and *Clostridium perfringens*.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Indicator strains of *Salmonella* enterica serovar Abony ATCC BAA-2162, *Salmonella* enterica serovar Typhimurium ATCC 14028, and *Salmonella* enterica serovar Choleraesuis ATCC 10708 were cultured in MRS broth and incubated aerobically at 37℃ for 24 h. *Clostridium perfringens* ATCC 13124 was cultured in fastidious anaerobic broth (Lab M, Heywood, UK) supplemented with (0.5%) yeast extract and incubated anaerobically for 24 h at 37℃. Isolated strains from this study were cultured in MRS broth supplemented with (0.1%) L-cysteine for 24 h at 37°C under aerobic or anaerobic (a gas mixture of 5% CO_2, 5% H_2, and 90% N_2; Whitley Anaerobe Systems A35, Don Whitley Scientific, UK) conditions. All isolated strains were purified three times on respective agar media plates. The strains were maintained in 50% glycerol stock at -80°C until use.

2.2. Preparation of ceca mucosal scrapings from the chicken intestinal tract

Broiler chicken intestinal tracts were obtained from a local slaughterhouse (Berube Poultry, Mountain, Canada) and Ringers solution (NaCl 7.2g/L, $CaCl_2$ 0.17g/L, and KCl 0.37g/L at pH 7.3) supplemented with (0.1%) L-cysteine (VWR, Ohio, USA) was poured over them and added to an anaerobic jar to preserve the microorganisms during transportation. All samples were processed upon arrival to the laboratory within an hour of collection. The intestinal tract was laid out, Ringers solution + (0.1%) L-cysteine sprayed over the tracts, and the mesentery, liver, heart, pancreas, and gallbladder were removed. Butchers twine was used to knot directly above and under the ceca to prevent the mixing of gut contents. The ceca were cut open, contents were removed, and then the mucosa was scrapped and added to a 15 mL centrifuge tube. Mucosal scrapings from both ceca were added to the same tube. The dissection equipment was cleaned and sterilized with 70% Ethanol before scrapping off the mucosa. All intestinal tracts were screened for *Salmonella* infection using MacConkey Agar.

2.4. Determination of Antibacterial Activity

2.4.1. Screening using double layer technique

The protocol was modified from (Lo Verso et al., 2018). Briefly, 9 mL of de Man, Rogosa, and Sharpe (MRS) broth (Criterion, Hardy Diagnostics, CA, USA) supplemented with (0.l%) L-cysteine was added to the 15 mL centrifuge tube containing ceca mucosal scrapings, vortexed to ensure mixing, and then 10-fold serial dilutions were performed. Aliquots of 100 μL were spread onto either 1.2% MRS agar or Tryptic soy agar (TSA) (1.2%) (Criterion, Hardy Diagnostics, CA, USA) supplemented with (0.5%) Yeast extract (YE) (Biobasic, NY, USA), seeded with 1% *Salmonella* Abony ATCC BAA-2162. One set of Petri plates were incubated aerobically, and the other set was incubated anaerobically at 37°C for 48 h or 72 h, respectively. Those colonies that yielded inhibition zones were selected and cultured in MRS broth + (0.1%) L-cysteine for 24 h to be used for a spot-on lawn method as a first means of screening.

2.4.2. Spot-on lawn method

The protocol was adapted from (Hanchi et al., 2014). Briefly, an aliquot of 3μL of an overnight culture of isolated strains were spotted onto the surface of MRS (1.2%) agar or TSBYE (1.2%) agar pre-seeded with 1% overnight culture from *Salmonella* Abony ATCC BAA-2162 and incubated either aerobically or anaerobically at 37°C for 24 h. Those strains that showed inhibition zones were selected, purified three times by streaking on their respective media and, stock solutions prepared and maintained at -20°C and stored at -80°C in their represented media containing 50% volume per volume (v/v) glycerol (1:1).

2.4.3. Agar well-diffusion method

The protocol was adapted from (Hammami et al., 2009). Briefly, 25 mL of sterile MRS Agar (1.2%) was seeded with 1% *Salmonella* Abony ATCC BAA-2162, poured into a sterile petri dish, and allowed to solidify at room temperature for 30 min. Afterward, the wide end of a 5 mL pipette was used to create wells in the agar, and 80 μL of cell free supernatant (CFS) or ciprofloxacin (control) was added to the wells. The plates were stored at 4°C for 1 h to allow the CFS to diffuse through the agar and then incubated aerobically at 37°C for 24 h. The diameter of the inhibition zone was measured. The plates were prepared in duplicate and repeated with the other indicator strains. The most active isolated strains and indicator strain were chosen for the critical-dilution assay.

2.4.4. Critical dilution Microplate

Bacteriocin activity was determined using the microtitration method adapted from (Hanchi et al., 2014). Briefly, in a 96-well flat-bottomed plate (VWR, PA, USA), 125 μL of CFS was added to MRS broth and used to perform 2-fold serial dilutions, which were then seeded with 50 μL of 10^6 CFU/mL of *Salmonella* Typhimurium ATCC 14028. The plates were incubated for 24 h and absorbance at 600 nm was measured every 20 min using TECAN Magellan Microplate Reader (Spark, Austria).

2.5. Identification of isolated strains by 16S rDNA sequencing

The active strains were identified by 16S rRNA. Genomic DNA was extracted from the overnight culture of isolated strains using a NucleoSpin Microbial DNA kit (Macherey-

Nagel, Duren, Germany) as per manufacturer's instructions. The DNA's purity was then determined using TECAN NanoDrop by comparing the absorbance ratio at 260nm to 280nm (SPARK, Austria).

The 16S rRNA gene was amplified by PCR thermal cycler (Eppendorf, Germany) using 1391-R (5'-GACGGGCGGTGTGTR) and Bact-8F (5'-AGAGTTTGATCCTGGCTCAG-3') the universal primers (Invitrogen), in a total volume of 50 μL (Turner et al., 1999). The master mix contained 5 μL 10x PCR buffer (Invitrogen), 1.5 μL $MgCl_2$ (Invitrogen), 1 μNTPS (Invitrogen), 0.2 μL Taq polymerase (Invitrogen), 36.3 μL H_2O, 2.5 μL of each primer, and 1 μL of bacterial DNA. The thermal cycler program consisted of an initial hold at 95℃ for 5 min for denaturation and polymerase activation, 30 cycles of 94℃ for 30 sec, 55℃ for 30 sec, 72℃ for 1 min 30 sec, and a final elongation step of 5 min at 72℃. PCR products were then used to perform gel electrophoresis (110V, 45 min) on a 1% agarose gel in 1x Tris-acetate-EDTA buffer and visualized using DNA gel loading dye (Invitrogen). The PCR products were then purified using a QIAquick PCR purification kit (Qiagen, Germany) and sequenced by the Ottawa Hospital Research Institute's DNA sequencing facility (Ottawa, Ontario, Canada). The resulting sequences were compared with the sequences in the GenBank database using the BLAST program. The criterion used to identify an isolate to the species level was having an identity greater than 99% in the 16S rRNA gene sequence.

2.6. Safety evaluation and in vitro probiotic potential

2.6.1. Antibiotic susceptibility test

The protocol was adapted from (Hanchi et al., 2014). Briefly, in a 96-well flat-bottomed plate, 100 μL of twice the selected antibiotic concentration was added to 100 μL of MRS broth with (0.1%) L-cysteine and used to perform 2-fold serial dilutions. The wells were then seeded with 100 µL of 10^5 CFU/mL of the selected bioactive strains. The absorbance at 600 nm was measured using TECAN Magellan Microplate Reader (Spark, Austria) at 0 hr and then incubated in the anaerobic chamber for 24 hours, then absorbance was read once again. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of the antibiotic that will inhibit the visible growth of the microorganism. The antibiotics tested were ampicillin, vancomycin, gentamycin, streptomycin, erythromycin, tetracycline, and chloramphenicol (All antibiotics were obtained from Alfa Aesar, Mississauga, Canada apart from gentamycin, which was from VWR, New York, USA).

2.6.2. Tolerance to bile salts and gastric acidity

The protocol was adapted from (X. Yang et al., 2014). Briefly, simulated gastric juice (SGJ) and intestinal juice (SIJ) was prepared according to the United States Pharmacopoeia (USPCCE 2004) (Q. Wang et al., 2009). SIJ consisted of 10g L^{-1} pancreatin (Wards Science, St Catherines, Canada) dissolved in 0.05 mol L^{-1} KH_2PO_4, at pH 7·4, and SGJ consisted of 2g L^{-1} pepsin (Wards Science, St Catherines, Canada) and 2g L^{-1} NaCl at pH 1·5 (Q. Wang et al., 2009). The SIJ was used to test bile salts tolerance of isolated strains, and the SGJ was used to test tolerance to pH. To the SIJ, oxgall bile salts (VWR, Mississauga, Canada) were added at varying concentrations of 0.3%, 0.6%, 0.8%, 1%, and 1.5% to mimic the physiological concentrations of the intestinal tract (X. Yang et al., 2014). The SGJ was divided into centrifuge tubes with a pH of 2.6 and 4.5, the gizzard's pH, and the crop, respectively (Chang & Chen, 2000). The pH was adjusted using 5 N NaOH or 1 M HCl. All fluids were filter sterilized with 0.45 µm filter.

A 96-well flat-bottomed plate was used to determine the tolerance to bile salts. 100 μL of intestinal juice with different bile salt concentrations was added to the wells, in triplicate followed by 100 μL of isolated strains. The resulting 200 μL was mixed with a multichannel pipette. Enumerations using the drop plate method were performed at 0, 3, 6, and 24 h. The plate was incubated anaerobically at 37℃. Similarly, the tolerance to pH was tested using 100 μL of pH adjusted gastric juice seeded with 100 μL of isolated strains cultured for 18 h. Enumeration using the drop plate method was performed at 0 h, 15 min, 30 min, and 90 min. The controls consisted of intestinal juice or gastric juice with no digestive enzymes. The assay was performed in triplicates.

2.7. Statistical Analysis

All experiments were carried out in triplicate. Results were log-transformed. Graph pad Prism 8 was used to perform statistical analysis. A p-value <0.05 was considered to be significant. One-way ANOVA was used to compare control and treatment samples in the bile salt tolerance test. A paired t-test was used to compare control to treatment samples in the pH tolerance test. Dunnett's multiple comparisons test was used to analyze further results obtained from ANOVA and t-test.

3. Results

3.1. Screening and isolation of bioactive strains and characterization of their inhibitory activity

A total of 290 colonies were initially selected based on inhibition zones, from all four intestinal tracts obtained from a local slaughterhouse. Screening for the presence of *Salmonella* was carried out on MacConkey agar plates, and all intestines were free from *Salmonella* infection. The samples were first screened by spot-on the lawn method (data not shown). Of the initial 290 colonies, the number of active isolated strains were narrowed down to 72 isolates that showed activity against *Salmonella* Abony ATCC BAA-2162. This step was followed by another round of screening using agar well diffusion, which narrowed down our active colonies from 72 to 55 isolates showing strong inhibition of *Salmonella* Abony ATCC BAA-2162. Next, we tested the cell-free supernatants (CFS) of the 55 isolates against *C. perfringens* ATCC 13124, *S.* enterica serovar Abony ATCC BAA-1262, *S.* enterica serovar Typhimurium ATCC 14028, *S.* enterica serovar Choleraesuis ATCC 10708, and *E. coli* ATCC 25922. Figure 2.1 illustrates the inhibition of indicator strains by selected strains' CFS, while the inhibitions' diameter is summarized in Table 2.1. As shown in Figure 2.2, the CFS of colonies A12, A19, A27, T3, and T8A induced a dose-dependent inhibition of *Salmonella* Typhimurium ATCC 14028 over 24 h of incubation. The CFS of the five strains had similar antimicrobial potency and completely inhibited the growth of *Salmonella* at 1×, ½, and ¼, but induced partial inhibition at higher dilutions.

3.2. Molecular identification of isolated bioactive strains

The most active strains were sequenced using 16S rRNA. The isolates belonged to *Ligilactobacillus salivarius* (n=19), *Ligilactobacillus agilis* (n=1), *Lactobacillus johnsonii* (n=7), *Lactobacillus kitasatonis* (n=1), *Lactobacillus sp*. (n=2), uncultured bacterium (n=1). The identity was based on a partial sequence match in the GenBank database with those isolates having an identity greater than 99% in the 16S rRNA gene sequence. The strains selected were T3 identified as *Ligilactobacillus salivarius*, T8a identified as *Ligilactobacillus agilis*, A12 identified as *Lactobacillus kitasatonis*, and A19 identified as

Ligilactobacillus salivarius (Table 2.2). The molecular identification of the strain A27 revealed a new uncultured bacterium with partial similarity to *Lactobacillus gallinarum*.

3.3. Probiotic potential of bioactive strains

The breakpoints cut-off values were determined from the EFSA panel on additives and products or substances used in animal feed (EFSA, 2012), and MIC summarized in Table 2.3. *L. agilis* T8a and *L. kitasatonis* A12 breakpoint cut-off values were assumed to be the same as *L. salivarius;* all four are facultative homofermentative species. All tested strains showed susceptibility to ampicillin, with *L. kitasatonis* A12 and uncultured bacterium A27 showing susceptibility to vancomycin as well. All isolates showed resistance to gentamycin, streptomycin, erythromycin, tetracycline, and chloramphenicol.

The selected strains were subjected to acidic and pH bile salts under simulated gastric intestinal and juices to determine the viability of our isolated strains to harsh gastrointestinal conditions. For the pH tolerance test, simulated gastric juice with a pH of 2.6 and 4.5 was selected to represent the physiological pH of the gizzard and the crop segments, respectively. The selected strains had a high survival rate at both pH 4.5 and 2.6 with a respective range of 88.97-99.33% and 76.80-93.98% (Figure 2.3). The strains *L. kitasatonis* A12, *L. salivarius* A19, and uncultured bacterium A27 exhibited that highest survival rate, while *L. agilis* T8A was the most sensitive to both tested pH. For the bile salts tolerance test, varying concentrations of oxgall bile salts were added to the simulated intestinal juice, containing pancreatin. The bile salts concentrations tested included 0.3%, 0.6%, 0.8%, 1%, and 1.5%. The lowest concentration showing bacterial growth was at 0.3% bile salts (Figure 2.4), while no growth of tested strains was observed at higher concentrations. The difference between the control and the treated samples was statistically significant at *p-value* (<0.05).

4. Discussion

Probiotics have shown to modulate the immune system, increase the proliferation of beneficial commensal bacteria, increase nutrient absorption and hence increase feed conversion efficiency, and increase body weight (Suresh et al., 2018). For some probiotics, such as *Lactobacilli*, these beneficial properties are due to the production of bacteriocins and short-chain fatty acids. *Lactobacilli* and other anaerobic bacteria are found to adhere to the epithelium of the intestinal tract, which is also abundant with *Bifidobacterium* sp., *Enterococcus faecium*, and *Pediococcus* spp., with the ceca having the highest concentration of anaerobic bacteria (Micciche et al., 2018b). FAO and WHO recommend that a probiotic be able to survive the passage, adhere, and multiply through the GIT, be a Gram-positive organism, show measurable health benefits, and have a defined dosage duration (Patterson, 2008; M. L. Wan et al., 2018). In this study, we aimed to isolate potential probiotic bacterial strains from the mucosa of the ceca from broiler chickens that could reduce *Salmonella and C. perfringens*, important foodborne pathogens. We chose to target the mucosa of the intestinal tract rather than the lumen contents because the commensal strains are already ideally suited to that specific environment via adhesion and competitive exclusion, therefore, have a head start at potential probiotic properties (Adhikari & Kwon, 2017b).

To accelerate the isolation and screening of potential probiotic strains, instead of allowing the colonies to grow on MRS agar plates for 24-48 hours per standard protocol, we adapted the protocol to simultaneously allow the growth of colonies while they exhibited inhibition zones on the agar plates. A total of 290 presumably active isolates, aerobic and anaerobic, from four intestines were selected based on the presence of inhibition zones in the initial double agar method. These colonies were further screened using the spot-on lawn method then agar well diffusion assay, resulting in 55 inhibitory strains against *Salmonella*. Additionally, dose-dependent inhibition of *Salmonella* over 24 hours was observed in all tested strains, which might be attributed to the presence of short-chain fatty acids (lactic acid, propionate, butyrate, and acetate), hydrogen peroxide, or bacteriocin-like compounds. For instance, selected bioactive strains belong to the lactobacilli group, members of the lactic acid bacteria, a broadly defined group characterized by lactic acid production as a sole or main end product from carbohydrate fermentation (Pan & Yu, 2013a).

The predominant identity of the isolated strains was from *L. salivarius,* followed by *L. johnsonii*. We also isolated and identified other *lactobacilli* strains belonging *to L. agilis*, *L. kitasatonis*, *Lactobacillus* sp., and a new uncultured bacterium. This is consistent with findings that the *L. acidophilus* group (*L. crispatus, L. gallinarum, and L. johnsonii*), *L. agilis, L. salivarius*, and *L. reuteri* are commonly present *Lactobacilli* in chicken intestines (Lan et al., 2003). From among the *L. acidophilus* group, it was found that *L. salivarius* was the predominant species among the intestinal microbiota of young chickens, which is consistent with what we found from our isolation results (Lan et al., 2003). An interesting find that *L. kitasatonis* was originally isolated from the intestinal tract of chickens in Japan in 2003 (Mukai et al., 2003). Since then, *L. kitasatonis* has been isolated from dogs in Japan (S.-Y. Kim et al., 2006), geese feces in Poland (Dec et al., 2014), and pig feces in Italy (De Angelis et al., 2006). Different species are found along the chicken intestinal tract. *Lactobacilli* species are found in higher concentrations in the upper GIT but also in the ileum than cecum (Adhikari & Kwon, 2017b). It has also been reported that *L. salivarius* and *L. johnsonii* are found in a higher percentage in the ileal mucosa compared to cecal mucosa or cecal lumen, but there was no statistical difference between them (Adhikari & Kwon, 2017b). The same researchers found *L. salivarius* to be a predominant species along with the entire GIT, which is consistent with other reports that in chickens aged 36-days-old; *L. salivarius* has a higher percentage of being isolated from both the ileum and cecum (Adhikari & Kwon, 2017b).

All isolated strains showed susceptibility to ampicillin but resistance to vancomycin, gentamycin, streptomycin, erythromycin, tetracycline, and chloramphenicol. Lactobacilli are considered non-pathogenic and are widely used as probiotics and starter cultures for various foods, supported by a long history of safe usage (Campedelli et al., 2018). Despite their safety status, many Lactobacillus species have been reported as intrinsically resistant to antibiotics, such as vancomycin, gentamicin, kanamycin, streptomycin, and ciprofloxacin, but susceptible to penicillin and β-lactams (Campedelli et al., 2018). Further investigation of this resistance's nature, whether chromosome-mediated or plasmid-mediated, is necessary, which could be done using whole-genome sequencing.

Resistance to harsh gastrointestinal conditions is important during probiotic selection. Our selected strains exhibited a high survival rate at acidic conditions mimicking the physiological pH of crop and gizzard, indicating their suitability as potential probiotics as they would be able to survive passage through the GIT and attach to the mucosa via competitive exclusion (the strains were originally isolated from the mucosa of the ceca). Our results showed superior results to those reported by (Yamazaki et al., 2012), whose *L. salivarius* and *L. kitasatonis* strains could not survive the acid tolerance test after one-hour incubation. Our selected strains were not able to survive tested bile salt concentrations higher than 0.3%. Similar results were obtained by (Yamazaki et al., 2012), who observed that only a single *L. kitasatonis* strain was able to survive growth in 0.3% oxgall bile salts. While our strains were unable to remain viable past 0.3% oxgall bile salt concentration, it by no means disqualifies our strains as potential probiotic candidates. Indeed, the actual bile salt concentration in chicken duodenum, jejunum, and cecum is 1.75mg/mL, 7mg/ml, and 0.085mg/mL, respectively (Lin et al., 2003). Our strains were able to survive at the lowest tested oxgall concentration of 3mg/mL, indicating that they would survive passage to the duodenum and ceca if well formulated.

5. Conclusion

The present study provides evidence that lactobacilli strains isolated from chicken caecal mucosa produce inhibitory substances against *Salmonella* and *Clostridium perfringens*. While *L. salivarius* T3, *L. agilis* T8A, *L. kitasatonis* A12, *L. salivarius* A19, and a new uncultured bacterium presented some interesting probiotic features, further investigation is required toward their application as novel probiotic strains in the poultry industry.

Author Contributions

AL and RH were responsible for designing the study, selecting the methods, and editing of the paper. AL and YAC dissected and performed the first screening using double layer assay. AL was responsible for drafting the study.

Acknowledgements

This study was supported by a grant from the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) [No. OAF-2019-100380].

Table 2.1. Determination of the diameter of inhibition of indicator strains by cell-free supernatants (CFS) extracted from bioactive isolated strains in this study. The plates were incubated aerobically (Strains 9-T9B) or anaerobically (Strains 2-27) at 37 ℃ for 24 hr. The larger the diameter of zone of inhibition, the stronger the inhibitory effect of the CFS.

Strains ID	**Inhibition zone (mm)**				
	***Salmonella* Abony ATCC BAA-2162**	***Salmonella* Typhimurium ATCC 14028**	***Salmonella* Choleraesuis ATCC 10708**	***Escherichia coli* ATCC 25922**	***Clostridium perfringens* ATCC 13124**
9	15	12	14	10	10
18	14	15	11	10	12
23	11	13	12	6	11
24	12	16	-	10	7
34	10	19	-	10	8
37	11	15	13	10	10
T3	13	16	15	10	12
T4	12	15	10	10	11
T6	13	15	10	6	11
T9	13	15	11	10	11
T10	14	14	13	10	14
T11	11	15	10	10	12
T12	10	13	10	7	9
T18	10	14	13	9	11
T21	10	12	9	7	8
T22	15	12	11	10	12
T26	14	14	11	10	12
T30	15	13	10	6	10
T33	14	15	14	9	11
T35	14	14	10	9	11
T38	13	15	13	7	10
T39	13	15	13	7	12
T5A	15	14	10	7	11
T8A	13	15	10	8	10
T9A	12	13	10	7	11
T9B	13	15	10	8	11
2	12	15	15	-	14
3	-	12	15	-	13
5	-	14	12	-	12
6	-	14	14	0.7	13
8	-	12	14	-	13
12	11	13	14	-	13
13	-	12	17	-	14
19	11	13	15	0.9	14
22	-	13	10	-	13
23	-	12	14	-	14
24	-	16	13	0.8	14
25	-	14	14	-	14
27	-	14	13	-	14

Table 2.2. Molecular identification of isolated bioactive strains by 16S rRNA sequencing

Chicken	location/type	Anatomy	Sample Id	Identity
#3	mucosal/aerobic	ceca	9	*Ligilactobacillus salivarius*
#3	mucosal/aerobic	ceca	18	*Ligilactobacillus salivarius*
#4	mucosal/aerobic	ceca	T3	***Ligilactobacillus salivarius***
#4	mucosal/aerobic	ceca	T4	*Ligilactobacillus salivarius*
#4	mucosal/aerobic	ceca	T6	*Lactobacillus johnsonii*
#4	mucosal/aerobic	ceca	T9	*Lactobacillus* sp.
#4	mucosal/aerobic	ceca	T10	*Ligilactobacillus salivarius*
#4	mucosal/aerobic	ceca	T11	*Ligilactobacillus salivarius*
#4	mucosal/aerobic	ceca	T18	*Ligilactobacillus salivarius*
#4	mucosal/aerobic	ceca	T22	*Ligilactobacillus salivarius*
#4	mucosal/aerobic	ceca	T26	*Ligilactobacillus salivarius*
#4	mucosal/aerobic	ceca	T30	*Lactobacillus* sp.
#4	mucosal/aerobic	ceca	T33	*Ligilactobacillus salivarius*
#4	mucosal/aerobic	ceca	T35	*Lactobacillus johnsonii*
#4	mucosal/aerobic	ceca	T38	*Ligilactobacillus salivarius*
#4	mucosal/aerobic	ceca	T39	*Ligilactobacillus salivarius*
#1	mucosal/aerobic	ceca	T5A	*Ligilactobacillus salivarius*
#1	mucosal/aerobic	ceca	**T8A**	***Ligilactobacillus agilis***
#1	mucosal/aerobic	ceca	T9A	*Ligilactobacillus salivarius*
#1	mucosal/aerobic	ceca	T9B	*Ligilactobacillus salivarius*
#4	mucosal/anaerobic	ceca	A2	*Lactobacillus johnsonii*
#4	mucosal/anaerobic	ceca	A3	*Lactobacillus johnsonii*
#4	mucosal/anaerobic	ceca	A6	*Ligilactobacillus salivarius*
#4	mucosal/anaerobic	ceca	A8	*Lactobacillus johnsonii*
#4	mucosal/anaerobic	ceca	**A12**	***Lactobacillus kitasatonis***
#4	mucosal/anaerobic	ceca	A13	*Ligilactobacillus salivarius*
#4	mucosal/anaerobic	ceca	**A19**	***Ligilactobacillus salivarius***
#3	mucosal/anaerobic	ceca	A23	*Lactobacillus johnsonii*
#3	mucosal/anaerobic	ceca	A24	*Ligilactobacillus salivarius*
#3	mucosal/anaerobic	ceca	A25	*Lactobacillus johnsonii*
#3	mucosal/anaerobic	ceca	**A27**	**UNCULTURED bacterium**

Table 2.3. Minimum inhibitory concentration (MIC in μg/mL) and interpretation of selected strains. MIC's smaller than or equal to the breakpoint value are designated as Susceptible (S) to the corresponding antibiotic whereas MIC's greater than the breakpoint value are designated as Resistant (R).

Antibiotic		**T3**		**T8a**		**A12**		**A19**		**A27**	
	Breakpoint	***MIC***	***Susceptibility***	***MIC***	***Susceptibility***	***MIC***	***Susceptibility***	***MIC***	***Susceptibility***	***MIC***	***Susceptibility***
Ampicillin	4	2	S	2	S	2	S	4	S	2	S
Vancomycin	n.r	>32	R	>32	R	4	S	>32	R	4	S
Gentamicin	16	>32	R	>32	R	>32	R	>32	R	>32	R
Streptomycin	64	>32	R	>32	R	128	R	>32	R	128	R
Erythromycin	1	2	R	4	R	1	S	4	R	2	R
Tetracycline	8	>32	R	>32	R	>32	R	>32	R	8	S
Chloramphenicol	4	16	R	16	R	16	R	>32	R	16	R

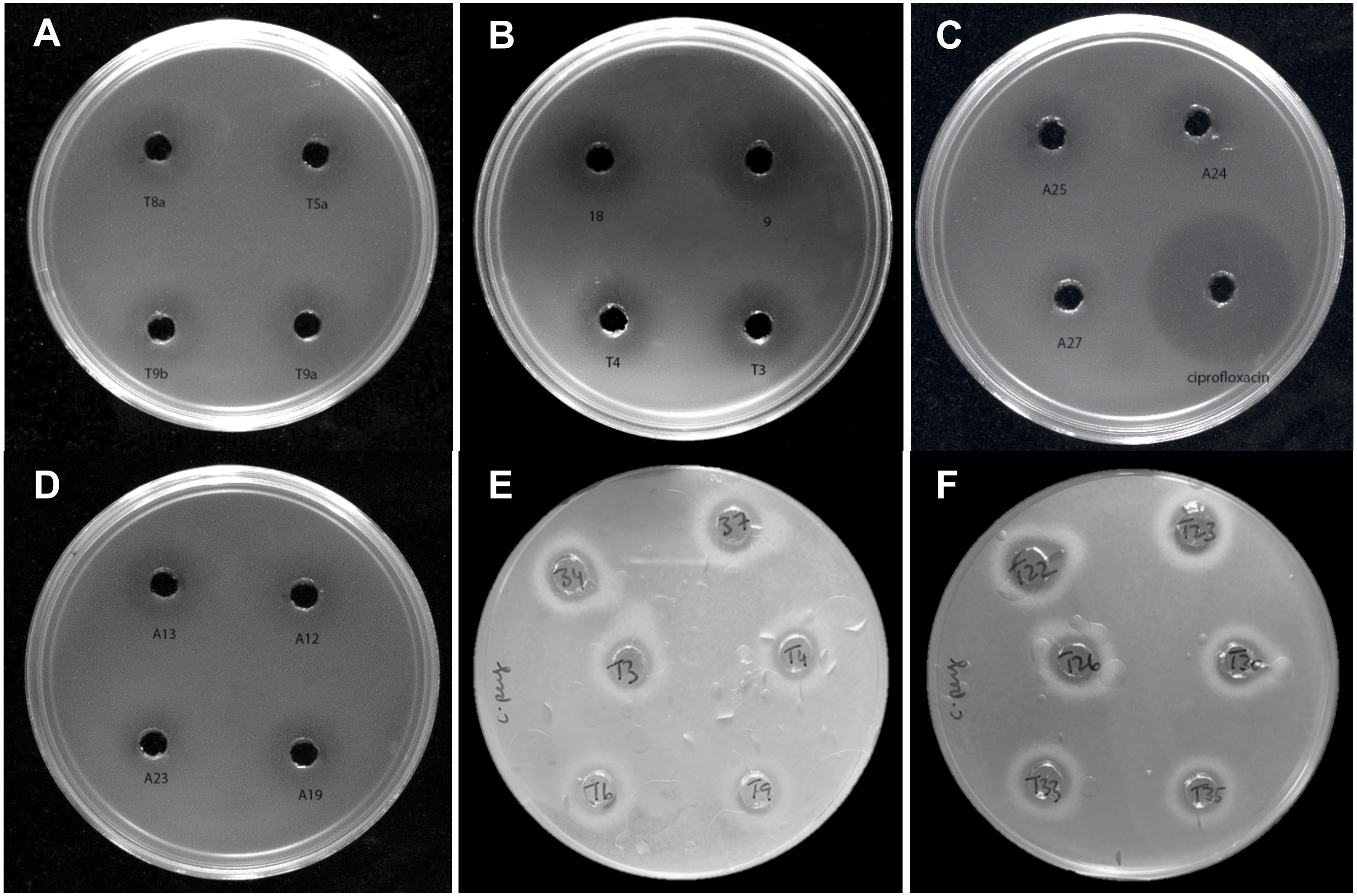

Figure 2.1. Agar well diffusion illustrating the growth inhibition of *Salmonella* Typhimurium ATCC 14028 (A-D) and *Clostridium perfringens* ATCC 13124 (E, F) by cell-free supernatants (CFS) extracted from the isolated strains. Ciprofloxacin was used as positive control.

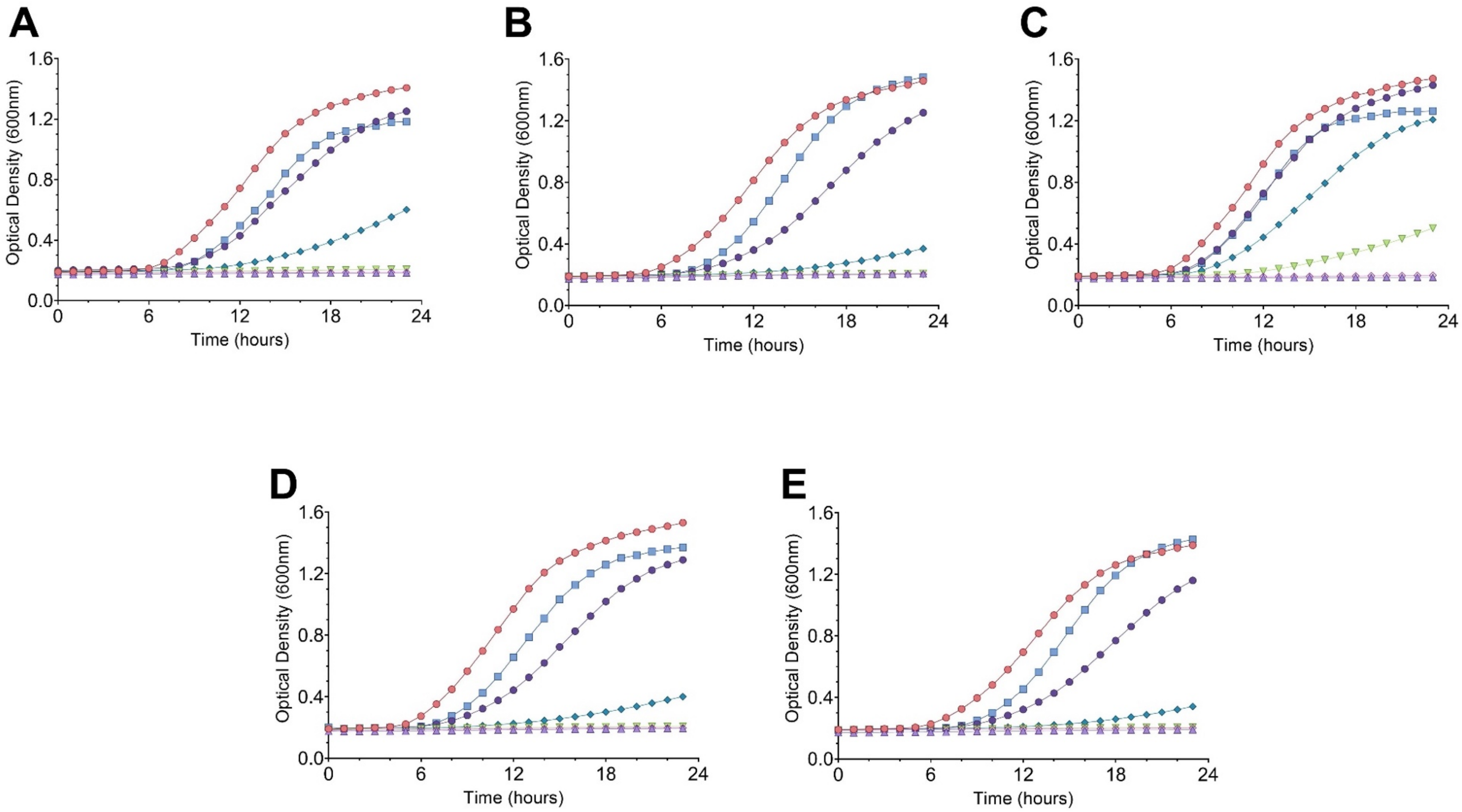

Figure 2.2. Dose-response growth inhibition of *Salmonella* Typhimurium ATCC 14028 by cell-free supernatants from strains A12 (A), A19 (B), A27 (C), T3 (D), and T8A (E). Tested concentrations are 1× (purple triangle), 1/2 (magenta diamond), 1/4 (triangle), 1/8 (diamond), 1/16 (dark purple circle), 1/32 (blue square), and 0 (negative control; red circle).

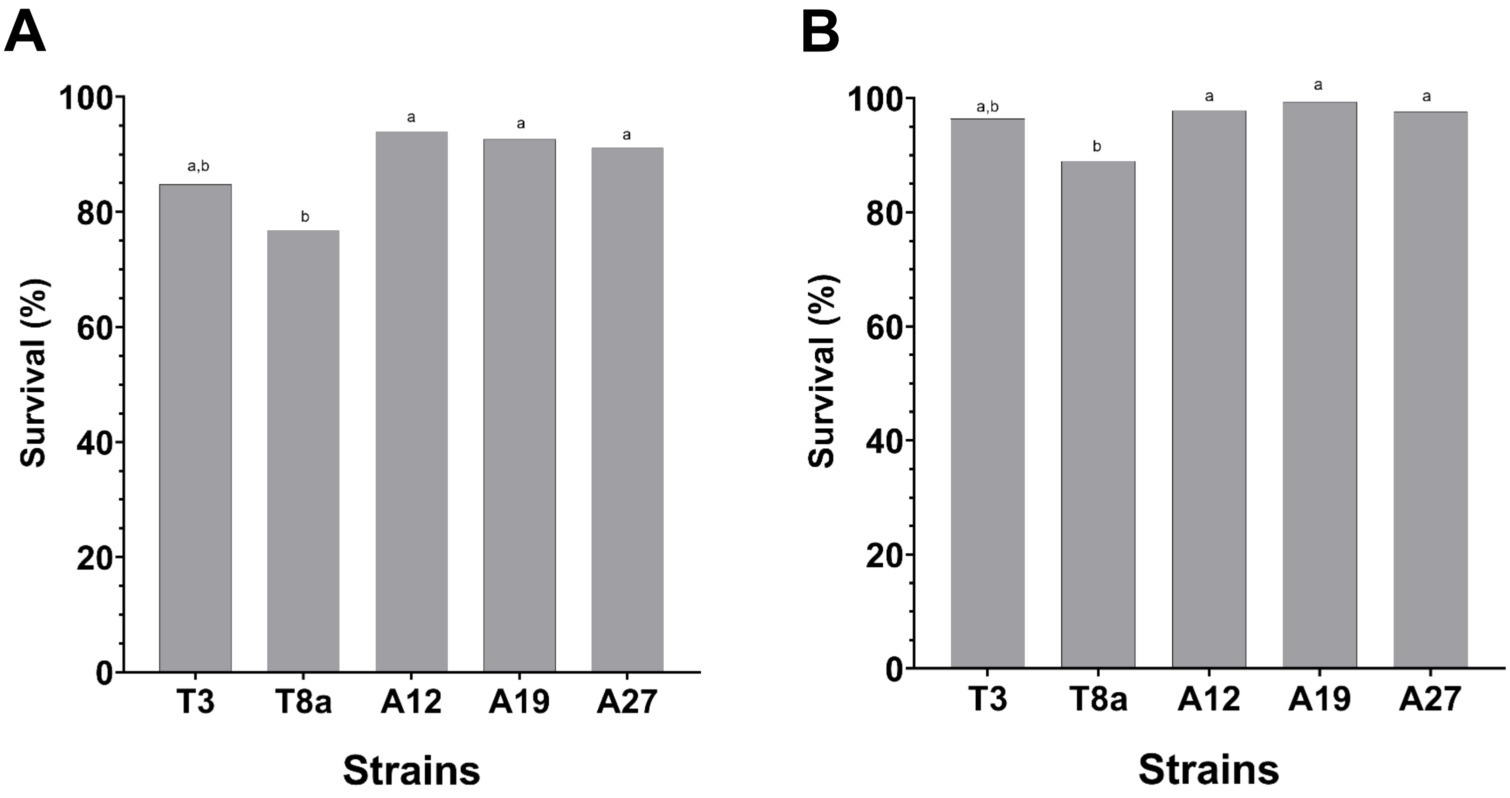

Figure 2.3. Percentage of survival of selected bioactive strains under acidic pH 2.6 (Gizzard; A) and pH 4.5 (Crop; B) in simulated gastric juice

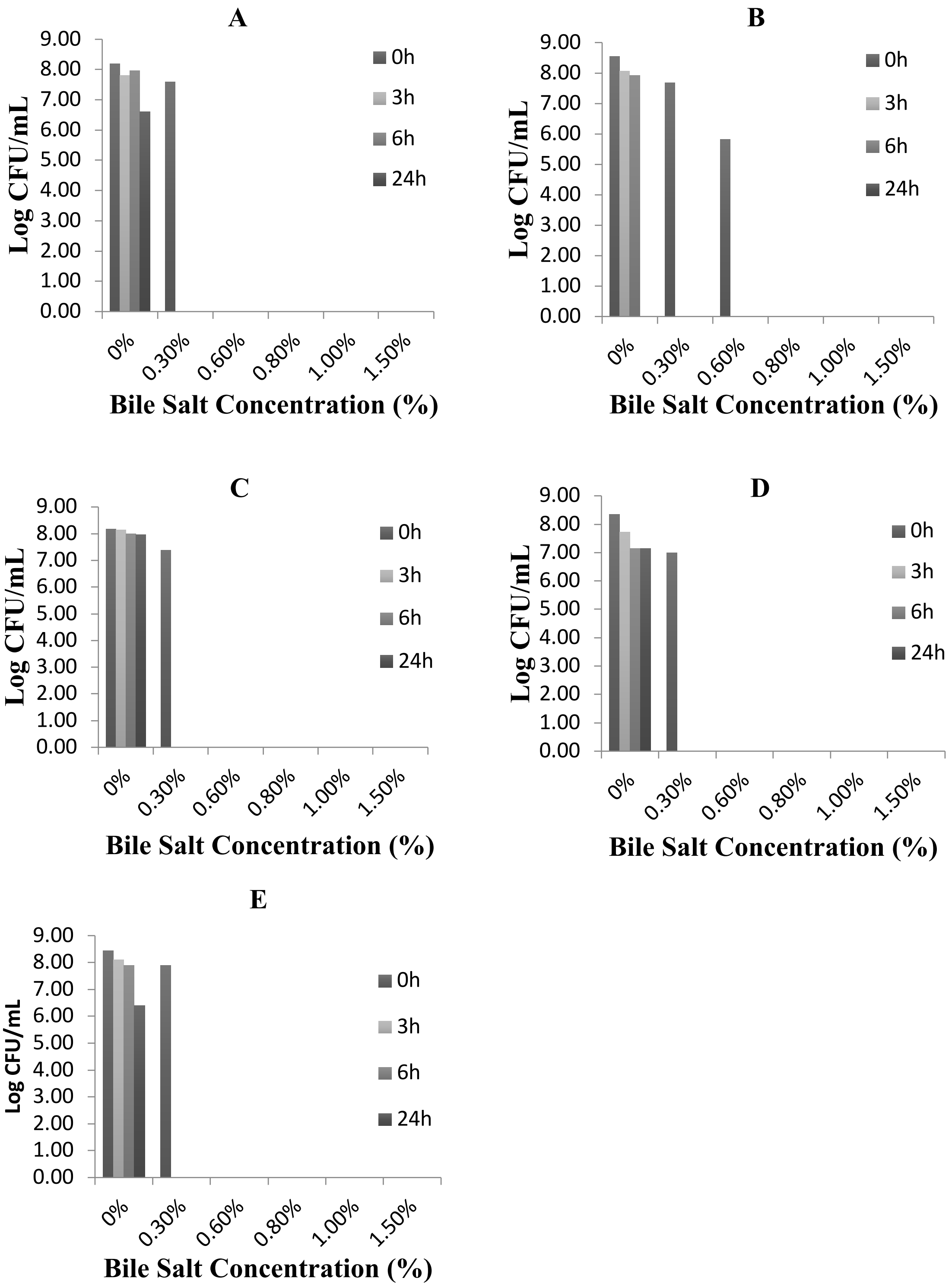

Figure 2.4. Isolated strains tested for bile salt tolerance in simulated intestinal juice at time 0, 3, 6, and 24 h. A (T3), B (T8a), C (A12), D (A19), E (A27). Asterix indicates statistical significance.

Chapter 3

Draft Whole-Genome Sequence of *Ligilactobacillus* and *Lactobacillus* species Isolated Strains from Chicken Mucosal Intestinal Tract of Broiler Chickens

Ayesha Lone[1], Walid Mottawea[1], Riadh Hammami[1, *]

[1] NuGUT Research Platform, School of Nutrition Sciences, Faculty of Health Sciences,
University of Ottawa, Ottawa, Ontario, Canada

* Corresponding author. E-mail address: riadh.hammami@uottawa.ca

Abstract

We isolated *Ligilactobacillus* and *Lactobacillus* species from the ceca mucosa of commercial broiler chickens and performed whole-genome sequencing on the most active strains inhibiting *Salmonella* and *Clostridium perfringens* using Illumina MiSeq. Four of the five strains have bacteriocin producing genes and lack plasmids, toxins, adhesion toxins, and transposable elements. This finding is useful for future *in vitro* and *in vivo* studies involving characterization and development of probiotics for poultry health.

Keywords: *Ligilactobacillus*, *Lactobacillus*, draft-genome, bacteriocin, probiotics

1. Introduction

The poultry gastrointestinal tract is shorter than other animals with differing pH and feed retention time (Micciche et al., 2018c). This particular physiology creates a diverse microbiome where the microorganisms have adapted to their unique environment. It is well known that the ceca have the highest concentration of anaerobic bacteria such as Lactobacilli (Micciche et al., 2018c). Lactobacilli are commonly used as probiotics due to their beneficial properties of producing bacteriocins and short-chain fatty acids. In our previous study, we isolated and identified potential probiotic strains from the ceca mucosa of commercial broiler chickens. This genomic study aims to confirm the safety and detect bacteriocin genes in isolated probiotic candidates.

2. Materials and methods

Broiler chicken intestinal tracts were obtained from a local slaughterhouse (Berube Poultry, Mountain, Canada). The ceca contents were removed, and the mucosa scrapped and added to de Man, Rogosa, and Sharpe (MRS) broth (Criterion, Hardy Diagnostics, CA, USA) supplemented with (0.l%) L-cysteine. Aliquots of 100μL were spread onto 1.2% MRS agar seeded with 1% *Salmonella* enterica serovar Abony ATCC BAA-2162. One set of Petri plates was incubated aerobically, and the other set was incubated anaerobically at 37°C for 48 hours or 72 hours, respectively. Those colonies that yielded inhibition zones were selected and cultured in MRS broth + (0.1%) L-cysteine for 24 hours. Genomic DNA was extracted from the overnight culture of isolated strains using a NucleoSpin Microbial DNA kit (Macherey-Nagel, Duren, Germany) as per manufacturer's instructions. The DNA's purity was then determined using TECAN NanoDrop by comparing the ratio of absorbance at 260nm to 280nm (SPARK, Austria). The PCR products were then purified using a QIAquick PCR purification kit (Qiagen, Germany). The isolates were identified using 16S rRNA sequencing, and the resulting sequences were compared with the sequences in the GenBank database using the BLAST program available on the National Center for Biotechnology Information website.

The DNA library was then prepared using the Nextera DNA Flex library prep kit according to the manufacturer's instructions. Briefly, the DNA is tagmented, index adapters added, and then amplified using PCR. The libraries are then purified, pooled, quantified for optimal cluster density, and then sequenced using the Illumina MiSeq platform (Illumina Inc., San Diego, USA). The data was then analyzed and annotated using FastQC v1.0.0 (Illumina BaseSpace Labs) and Rapid Annotations using Subsystems Technology (RAST) v2.0 using default settings (Aziz et al., 2008). BAGEL 4 and antiSMASH (Blin et al., 2019) were used to analyze bacteriocin genes' presence.

3. Results and discussion

Table 3.1 summarizes the identity and genomic information of the isolated strains as determined by BLAST and the organism overview obtained from RAST. Table 3.2 shows, in detail, all the bacteriocin genes that were identified, their location, and their similarity to BLAST search using both BAGEL 4 and antiSMASH. BAGEL 4 was able to identify the presence of bacteriocin genes in four of the five isolated strains with Enterolysin A being the commonly identified bacteriocin gene. *Lactobacillus kitasatonis* also had Helveticin-J genes, and *Ligilactobacillus salivarius* had salivaricin_P_chain_b genes. Likewise, antiSMASH identified lanthipeptide genes in *Lactobacillus kitasatonis* and Linocin_M18 genes in *Lactobacillus gallinarum. Ligilactobacillus salivarius* strain had salivaricin CRL1328 α peptide/salivaricin CRL1328 β peptide with 75% of genes showing similarity.

Our results show that our isolated strains have the capability to produce bacteriocins and lack plasmids, toxins, adhesion toxins, and transposable elements, they can be used in a future *in* *vitro* and *in vivo* trials to determine further their probiotic capability and effectiveness against poultry pathogenic strains.

Data availability

The whole genome sequence of all isolated strains will be deposited at GenBank under the BioProject accession number PRJNA685183.

Author Contributions

AL and RH were responsible for designing the study, selecting the methods, and editing of the paper. WM performed the WGS and AL analysed the data. AL was responsible for drafting the study.

Acknowledgements

This study was supported by a grant from the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) [No. OAF-2019-100380].

Table 3.1. Whole genome sequencing overview of isolated strains obtained from RAST server

Strain	**BLAST identity**	**Genome Size (bp)**	**GC content**	**Contig**	**N50**	**L50**	**Number of subsystems**	**Number of coding sequences**	**Number of RNAs**	**Closest neighbour**
T3	*Ligilactobacillus salivarius*	2,024,427	32.7	100	176765	4	211	1994	76	*L. salivarius* ATCC 11741
T8A	*Ligilactobacillus agilis*	2,345,855	41.0	110	116853	7	214	2346	92	*L. salivarius* ATCC 11741
A12	*Lactobacillus kitasatonis*	1,975,848	37.4	163	47569	16	189	2077	58	*L. delbruecki* subsp. bulgaricus ATCC 11842
A19	*Ligilactobacillus salivarius*	1,848,796	32.8	76	261149	2	212	1087	70	*L. salivarius* ATCC 11741
A27	Unculture bacterium	2,014,858	36.5	145	51519	15	192	2092	74	*L. acidophilus* NCFM

Table 3.2. Overview of all the bacteriocin genes that were identified, their location, and their similarity to BLAST search using both antiSMASH (A) and BAGEL 4 (B).

A

Strains	BLAST identity	Identified bacteriocin	Region	From	To	Similarity
T3	*Ligilactobacillus salivarius*	salivaricin CRL1328 α peptide/salivaricin CRL1328 β peptide	28.1	27,593	46,844	75%
A12	*Lactobacillus kitasatonis*	Lanthipeptide	29.1	1	19,666	
A27	Uncultured bacterium	Linocin_M18	15.1	33,984	44,931	

B

Strains	BLAST identity	Identified bacteriocin	NODE	From	To	Blast result
T3	*Ligilactobacillus salivarius*	63.3; Enterolysin A	8	149321	169768	49%
		153.2; MR10B	15	3548	19814	85.33%
		213.2; Salivaricin_P_chain_b	83	18395	51832	58.54%
		E64.3; Enterolysin A	1	165542	186031	38.93%
A12	*Lactobacillus kitasatonis*	70.3; Helveticin J	60	3635	15615	37.15%
		62.3; Enterolysin A	93	1	14236	62.73%
		64.3; Enterolysin A	93	11342	23788	81.22%
		6.3 Bacteriocin_Helveticin J	190	39656	56375	90.20%
A19	*Ligilactobacillus salivarius*	153.2; MR10B	6	112	16846	58.54%
		64.3; Enterolysin A	3	172138	192627	38.93%
A27	Uncultured bacterium	62.3; Enterolysin A	53	1739	22201	61.49%
		64.3; Enterolysin A	53	19412	40021	85.33%

Discussion

With the global bans on antibiotics as growth promoters and prophylactics, it is important to acknowledge that a one-size-fits-all program will not work for most poultry industries. To avoid severe economic losses due to production losses after the ban of antibiotics in Europe, the poultry industry must be looking forward to multi-pronged alternatives to antibiotics with a strong history of antimicrobial activity, safety, and low cost. It is equally important to improve the environmental and living conditions for the animals.

Providing a clean and improved living environment for the birds would be the first step in maintaining a healthy poultry gut microbiome, therefore, a healthy intestinal tract. A balanced microbiome is an important environment for probiotics to work effectively in a long-term manner due to the higher possibility of intestinal colonization and proliferation. Competitive gut colonization by probiotics ensures their production of beneficial compounds, including antimicrobial peptides that help maintain gut homeostasis. Hence the importance of selecting bacteriocin-producing probiotic strains by the industry. However, it is important to remember that probiotics' efficacy depends on several factors such as age and health of the bird, dose size, concentration, and administration. Thus, the importance and need for a multi-pronged approach. Care will have to be taken to optimize all of these factors before developing, formularizing, and commercializing our isolated strains as potential probiotics.

In this study, we achieved our aim of isolating mucosal commensal bacterial strains having strong inhibitory activity against both *Salmonella* Typhimurium and *C. perfringens*. Both of these pathogens, *C. perfringens* in particular, are responsible for high mortality rates in chickens. NE, a disease caused by *C. perfringens,* is often only diagnosed when chicken mortality occurs (Hofacre et al., 2018). This places an increased economic burden on the poultry industry. NE is also often subclinical and leads to increased feed conversion and weight loss, adding to the economic burden (Hofacre et al., 2018). 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.

During the screening process, we were also able to isolate an uncultured bacterium that shows similarity to *L. acidophilus.* One major reason for this success was the initial isolation process of scraping the mucosa for potential probiotic strains instead of the standard lumen contents. One of the properties of being considered a potential probiotic candidate is the ability to adhere to and colonize the mucosa via competitive exclusion. It was deduced that we would have a high probability of isolating potential probiotic strains that would be pre-disposed to attaching the mucosa, colonizing, and potentially proliferating. Additionally, a novelty of our project was performing our isolation and screening of potential probiotic strains simultaneously using an optimised double layer technique instead of the multi-day standard protocol. We were able to shorten the isolation and screening protocol by 48-72 hours instead of the standard 72 hours required for isolation and an additional 48-72 hours required for screening.

While we were not able to definitively relate the antimicrobial activity of our isolated strains to the bacteriocin-like activity in our assays, it is obvious that our isolated strains have bacteriocin producing genes, as seen with the whole genome sequencing results. Our results do not appear to be uncommon. In fact, (Nilsen et al., 2003) discussed that there appear to be some bacteriocinogenic strains that produce bacteriocins that can only be detected on solid media as opposed to the traditional isolation method of using culture supernatants, suggesting a regulated production. Further testing needs to occur to determine our isolated strains' ideal culture conditions to produce bacteriocins.

Our strains showed bacteriocin producing genes of Linocin M18, a bacteriocin that targets *Listeria* spp. (Hammami et al., 2010b), Salivarcin CRL1328 α/β originally produced by *L. salivarius* CR 1328 which was isolated from the human vagina (S. Messaoudi et al., 2013b), Helveticin J which is a type III chromosomally encoded bacteriocin produced by *Lactobacillus helveticus* 481 with a narrow spectrum of activity (Nilsen et al., 2003), and Lanthipeptide which is a class 1 bacteriocin that shows resistance to protease degradation (Ongey & Neubauer, 2016). Enterolysin A, another type III bacteriocin, was another bacteriocin whose genes were detected. This bacteriocin was originally isolated and purified from *Enterococcus faecalis* LMG2333 and is known to show a dose-dependent, broad-spectrum bacteriolytic activity against Gram-positive bacteria (Nilsen et al., 2003). This may explain our critical dilution microplate results where we observed a dose-dependent antibacterial response by the CFS of our isolated strains against *Salmonella* Typhimurium. Lastly, MR10B bacteriocin, which shows similarity to the L50A/L50B bacteriocins, genes were also detected (Martin-Platero et al., 2006).

Our research is not without its limitations. We could not determine the critical dilution microplate assay by the CFS of our isolated strains against *C. perfringens* under anaerobic conditions in the microplate reader. We also did not determine the nature of the antimicrobial activity against *Salmonella* Typhimurium and *C. perfringens* by the CFS of our isolates strains witnessed in both the agar well-diffusion assay as well as the critical dilution microplate assay. We have not been able to conclusively attribute the antimicrobial activity to bacteriocin-like activity or the presence of SCFAs or both. The antimicrobial effect of our isolated strains in a co-culture with *Salmonella* and with *C. perfringens* was also not determined. The nature of antibiotic resistance to erythromycin, tetracycline, and chloramphenicol was also not looked into. Additional probiotic potential genes such as genes for the presence of bile salt hydrolase was not determined.

Lastly, the concentrations of the bile salts tested to determine our isolated strains' tolerance did not mimic the actual concentrations found in the chicken gut.

Additional investigation on the probiotic potential of our isolates is necessary. For example, determining the nature of observed antibiotic resistance of our isolated strains to erythromycin, tetracycline, and chloramphenicol; determination of the presence of potential probiotic genes such as for the presence of adhesion and surface proteins, etc., in addition to the ability of our isolated strains to adhere to intestinal epithelial cells in the presence of *Salmonella* as well as *C. perfringens*. Further determination of our isolated strains' antibacterial activity against *C. perfringens* is also warranted. Optimization of growth conditions and characterization of the bacteriocins also need to be assessed. Before *in vivo* trials, simulated upper and lower gut trials involving a co-culture of our isolated strains with *Salmonella* and *C. perfringens* are of particular interest.

Our project shows interdisciplinarity in the way it is rooted in standard microbiology and molecular biology concepts and methods. It incorporates aspects of bioinformatics and adds to the literature on food safety and policy of commercialization of existing and novel bacteriocins.

Conclusion

Our aims to isolate and identify bacteriocin-producing bacterial strains obtained from the mucosa of the gastrointestinal tract of broiler chickens and determine the safety and probiotic characterization of isolated bacteria, both have been achieved. We have isolated multiple and diverse strains, including an uncultured bacterium with inhibiting activity against *Salmonella* Typhimurium ATCC 14028, *Salmonella* Abony ATCC BAA-1262, *Salmonella* Cholerasuis ATCC 10708, *Clostridium perfringens* ATCC 13124, and *E. coli* ATCC 25922. Our isolated strains show potential probiotic properties and the ability to tolerate acidic pH and bile salts. Our antibiotic susceptibility test results correlate with expected results for lactic acid bacteria, such as lactobacilli's inherent resistance to vancomycin, gentamycin, and streptomycin. Two of our isolated *Ligilactobacillus salivarius* strains may have additional properties for improving chicken phenotype, improved weight gain, carcass weight, and FCR, based on the abundant literature on *L. salivarius*. Finally, our strains show the presence of genes for bacteriocin production.

Taken together, the results of the present study provide evidence that lactobacilli and ligilactobacilli strains isolated from chicken ceca mucosa are able to inhibit both *Salmonella* and *Clostridium perfringens* and present some interesting probiotic features, that upon further investigation, would make excellent candidates towards their application as novel probiotic strains in the poultry industry.

Bibliography

Adhikari, B., & Kwon, Y. M. (2017a). Characterization of the Culturable Subpopulations of Lactobacillus in the Chicken Intestinal Tract as a Resource for Probiotic Development. *Frontiers in Microbiology*, *8*, 1389. https://doi.org/10.3389/fmicb.2017.01389

Adhikari, B., & Kwon, Y. M. (2017b). Characterization of the Culturable Subpopulations of Lactobacillus in the Chicken Intestinal Tract as a Resource for Probiotic Development. *Frontiers in Microbiology*, *8*, 1389. https://doi.org/10.3389/fmicb.2017.01389

Agyare, C., Etsiapa Boamah, V., Ngofi Zumbi, C., & Boateng Osei, F. (2019). Antibiotic Use in Poultry Production and Its Effects on Bacterial Resistance. In *Antimicrobial Resistance—A Global Threat*. IntechOpen. https://doi.org/10.5772/intechopen.79371

Alali, W. Q., & Hoface, C. L. (2016). Preharvest Food Safety in Broiler Chicken Production. In *Preharvest Food Safety* (pp. 69–86). American Society of Microbiology. https://doi.org/10.1128/microbiolspec.PFS-0002-2014

Anadón, A., Rosa Martínez-Larrañaga, M., & Aranzazu Martínez, M. (2006). Probiotics for animal nutrition in the European Union. Regulation and safety assessment. *Regulatory Toxicology and Pharmacology*, *45*(1), 91–95. https://doi.org/10.1016/j.yrtph.2006.02.004

Angelakis, E. (2017). Weight gain by gut microbiota manipulation in productive animals. *Microbial Pathogenesis*, *106*, 162–170. https://doi.org/10.1016/j.micpath.2016.11.002

Awad, W. A., Böhm, J., Razzazi-Fazeli, E., Ghareeb, K., & Zentek, J. (2006). Effect of Addition of a Probiotic Microorganism to Broiler Diets Contaminated with Deoxynivalenol on Performance and Histological Alterations of Intestinal Villi of Broiler Chickens. *Poultry Science*, *85*(6), 974–979. https://doi.org/10.1093/ps/85.6.974

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., … Zagnitko, O. (2008). The RAST Server: Rapid Annotations using Subsystems Technology. *BMC Genomics*, *9*(1), 75. https://doi.org/10.1186/1471-2164-9-75

Bajagai, Y. S., Klieve, A. V, Dart, P. J., & Bryden, W. L. (2016). *Probiotics in animal nutrition-Production, impact and regulation*.

Ben Lagha, A., Haas, B., Gottschalk, M., & Grenier, D. (2017a). Antimicrobial potential of bacteriocins in poultry and swine production. *Veterinary Research*, *48*(1), 1–12. https://doi.org/10.1186/s13567-017-0425-6

Ben Lagha, A., Haas, B., Gottschalk, M., & Grenier, D. (2017b). Antimicrobial potential of bacteriocins in poultry and swine production. *Veterinary Research*, *48*(1), 1–12. https://doi.org/10.1186/s13567-017-0425-6

Blin, K., Shaw, S., Steinke, K., Villebro, R., Ziemert, N., Lee, S. Y., Medema, M. H., & Weber, T. (2019). antiSMASH 5.0: Updates to the secondary metabolite genome mining pipeline. *Nucleic Acids Research*, *47*(W1), W81–W87. https://doi.org/10.1093/nar/gkz310

Campedelli, I., Mathur, H., Salvetti, E., Clarke, S., Rea, M. C., Torriani, S., Ross, R. P., Hill, C., & O'Toole, P. W. (2018). Genus-Wide Assessment of Antibiotic Resistance in *Lactobacillus* spp. *Applied and Environmental Microbiology*, *85*(1), e01738-18, /aem/85/1/AEM.01738-18.atom. https://doi.org/10.1128/AEM.01738-18

Canadian Food Inspection Agency. (2019). *Introduction-Regulation of livestock feed in Canada*. https://www.inspection.gc.ca/animals/feeds/regulatory-guidance/rg-1/introduction/eng/1493737712622/1493737821272

Card, R. M., Cawthraw, S. A., Nunez-Garcia, J., Ellis, R. J., Kay, G., Pallen, M. J., Woodward, M. J., & Anjum, M. F. (2017a). *An In Vitro Chicken Gut Model Demonstrates Transfer of a Multidrug Resistance Plasmid from Salmonella to Commensal Escherichia coli*. https://doi.org/10.1128/mBio.00777-17

Card, R. M., Cawthraw, S. A., Nunez-Garcia, J., Ellis, R. J., Kay, G., Pallen, M. J., Woodward, M. J., & Anjum, M. F. (2017b). An in Vitro chicken gut model demonstrates transfer of a multidrug resistance plasmid from Salmonella to commensal Escherichia coli. *MBio*, *8*(4), 1–15. https://doi.org/10.1128/mBio.00777-17

Cavera, V. L., Arthur, T. D., Kashtanov, D., & Chikindas, M. L. (2015). Bacteriocins and their position in the next wave of conventional antibiotics. *International Journal of Antimicrobial Agents*, *46*(5), 494–501. https://doi.org/10.1016/j.ijantimicag.2015.07.011

CDC. (2018a). *Clostridium perfringens*. Center for Disease Control and Prevention. https://www.cdc.gov/foodsafety/diseases/clostridium-perfringens.html

CDC. (2018b). *Outbreaks Involving Salmonella | CDC*. CDC. https://www.cdc.gov/salmonella/outbreaks.html

CDC. (2020, August 19). *Salmonella*. Center for Disease Control and Prevention. https://www.cdc.gov/salmonella/index.html

Center for Disease Control and Prevention. (2015a). *Serotypes and the Importance of Serotyping Salmonella | Salmonella Atlas | Reports and Publications | Salmonella | CDC*. Center for Disease Control and Prevention. https://doi.org/10.1208/s12249-010-9573-y

Center for Disease Control and Prevention. (2015b). *Serotypes and the Importance of Serotyping Salmonella | Salmonella Atlas | Reports and Publications | Salmonella | CDC*. Center for Disease Control and Prevention. https://www.cdc.gov/salmonella/reportspubs/salmonella-atlas/serotyping-importance.html#three

Chang, M. H., & Chen, T. C. (2000). Reduction of Campylobacter jejuni in a Simulated Chicken Digestive Tract by Lactobacilli Cultures. *Journal of Food Protection*, *63*(11), 1594–1597. https://doi.org/10.4315/0362-028X-63.11.1594

Chichlowski, M., Croom, W. J., Edens, F. W., McBride, B. W., Qiu, R., Chiang, C. C., Daniel, L. R., Havenstein, G. B., & Koci, M. D. (2007). Microarchitecture and spatial relationship between bacteria and ileal, cecal, and colonic epithelium in chicks fed a direct-fed microbial, PrimaLac, and salinomycin. *Poultry Science*, *86*(6), 1121–1132. https://doi.org/10.1093/ps/86.6.1121

Chikindas, M. L., Weeks, R., Drider, D., Chistyakov, V. A., & Dicks, L. M. (2018a). Functions and emerging applications of bacteriocins. In *Current Opinion in Biotechnology*. https://doi.org/10.1016/j.copbio.2017.07.011

Chikindas, M. L., Weeks, R., Drider, D., Chistyakov, V. A., & Dicks, L. M. (2018b). Functions and emerging applications of bacteriocins. *Current Opinion in Biotechnology*, *49*, 23–28. https://doi.org/10.1016/j.copbio.2017.07.011

Chugunov, A., Pyrkova, D., Nolde, D., Polyansky, A., Pentkovsky, V., & Efremov, R. (2013). Lipid-II forms potential “landing terrain” for lantibiotics in simulated bacterial membrane. *Scientific Reports*, *3*. https://doi.org/10.1038/srep01678

Coller, J. (2019). *Improving Antibiotics Stewardship in Livestock Supply Chains* (Engagement Progress Report). https://www.fairr.org/article/improving-antibiotics-stewardship-in-livestock-supply-chains/

Crhanova, M., Karasova, D., Juricova, H., Matiasovicova, J., Jahodarova, E., Kubasova, T., Seidlerova, Z., Cizek, A., & Rychlik, I. (2019). Systematic Culturomics Shows that Half of Chicken Caecal Microbiota Members can be Grown in Vitro Except for Two Lineages of Clostridiales and a Single Lineage of Bacteroidetes. *Microorganisms*, *7*(11), 496. https://doi.org/10.3390/microorganisms7110496

Crisol-Martínez, E., Stanley, D., Geier, M. S., Hughes, R. J., & Moore, R. J. (2017). Understanding the mechanisms of zinc bacitracin and avilamycin on animal production: Linking gut microbiota and growth performance in chickens. *Applied Microbiology and Biotechnology*, *101*(11), 4547–4559. https://doi.org/10.1007/s00253-017-8193-9

Cvm, F. (2017). *2016 Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals*.

De Angelis, M., Siragusa, S., Berloco, M., Caputo, L., Settanni, L., Alfonsi, G., Amerio, M., Grandi, A., Ragni, A., & Gobbetti, M. (2006). Selection of potential probiotic lactobacilli from pig feces to be used as additives in pelleted feeding. *Research in Microbiology*, *157*(8), 792–801. https://doi.org/10.1016/j.resmic.2006.05.003

Dec, M., Urban-Chmiel, R., Gnat, S., Puchalski, A., & Wernicki, A. (2014). Identification of Lactobacillus strains of goose origin using MALDI-TOF mass spectrometry and 16S–23S rDNA intergenic spacer PCR analysis. *Research in Microbiology*, *165*(3), 190–201. https://doi.org/10.1016/j.resmic.2014.02.003

Dexian, Z., Li, R., & Li, J. (2012). Lactobacillus reuteri ATCC 55730 and L22 display probiotic potential in vitro and protect against salmonella-induced pullorum disease in a chick model of infection. In *Lactobacillus: Classification, Uses and Health Implications* (Vol. 93, Issue 1, pp. 325–342). https://doi.org/10.1016/j.rvsc.2011.06.020

Dimier-Poisson, I. H., Bout, D. T., & Quéré, P. (2004). Chicken Primary Enterocytes: Inhibition of *Eimeria tenella* Replication After Activation with Crude Interferon-γ Supernatants. *Avian Diseases*, *48*(3), 617–624. https://doi.org/10.1637/7180-031604R

Ding, J., Dai, R., Yang, L., He, C., Xu, K., Liu, S., Zhao, W., Xiao, L., Luo, L., Zhang, Y., & Meng, H. (2017). Inheritance and establishment of gut microbiota in chickens. *Frontiers in Microbiology*, *8*(OCT). https://doi.org/10.3389/fmicb.2017.01967

Drider, D., Belguesmia, Y., Choiset, Y., Prévost, H., Dalgalarrondo, M., & Chobert, J. M. (2010). Partial purification and characterization of the mode of action of enterocin S37: A bacteriocin produced by enterococcus faecalis S37 isolated from poultry feces. *Journal of Environmental and Public Health*, *2010*. https://doi.org/10.1155/2010/986460

EFSA. (2012). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA Journal*, *10*(6). https://doi.org/10.2903/j.efsa.2012.2740

Eloit, M., Silva, J. G. da, & Chan, M. (2016). *WHO | Superbugs: Why we need action now*. WHO; World Health Organization. http://www.who.int/mediacentre/commentaries/superbugs-action-now/en/

European union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food 2016 published. (2018). In *European Food Safety Authority* (Vol. 16, Issue 2). https://doi.org/10.2903/j.efsa.2018.5182

Fields, O. F. (1996). Use of Bacteriocins in Food: Regulatory Considerations. *Journal of Food Protection*, *59*(13), 72–77. https://doi.org/10.4315/0362-028X-59.13.72

Flanagan, R. C., Neal-McKinney, J. M., Dhillon, A. S., Miller, W. G., & Konkel, M. E. (2009). Examination of Campylobacter jejuni Putative Adhesins Leads to the Identification of a New Protein, Designated FlpA, Required for Chicken Colonization. *Infection and Immunity*, *77*(6), 2399–2407. https://doi.org/10.1128/IAI.01266-08

Flint, J. F., & Garner, M. R. (2009). Feeding beneficial bacteria: A natural solution for increasing efficiency and decreasing pathogens in animal agriculture. *Journal of Applied Poultry Research*. https://doi.org/10.3382/japr.2008-00133

Food and Drug Administration. (2013). *Phasing out certain antibiotic use in farm animals*. https://www.fda.gov/consumers/consumer-updates/phasing-out-certain-antibiotic-use-farm-animals

Food and Drug Administration. (2018a). *Antimicrobial Resistance Information from FDA*. FDA. https://www.fda.gov/EmergencyPreparedness/Counterterrorism/MedicalCountermeasures/MCMIssues/ucm620149.htm

Food and Drug Administration. (2018b). *Supporting antimicrobial stewardship in veterinary settings* (Issue September 2018).

Food and Drug Administration. (2019). *Product regulation*. https://www.fda.gov/animal-veterinary/animal-food-feeds/product-regulation

Food, E., Authority, S., & EFSA. (2018). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2016. *EFSA Journal*, *16*(2). https://doi.org/10.2903/j.efsa.2018.5182

Food Safety and Standards Authority of India. (2019). *Direction Colistin Ban* (p. 2).

Gaucher, M.-L., Perron, G. G., Arsenault, J., Letellier, A., Boulianne, M., & Quessy, S. (2017). Recurring Necrotic Enteritis Outbreaks in Commercial Broiler Chicken Flocks Strongly Influence Toxin Gene Carriage and Species Richness in the Resident Clostridium perfringens Population. *Frontiers in Microbiology*, *8*, 881. https://doi.org/10.3389/fmicb.2017.00881

Government of Canada. (2017). *Antimicrobial resistance and animals-actions*. https://www.canada.ca/en/public-health/services/antibiotic-antimicrobial-resistance/animals/actions.html

Government of Canada. (2018a). *Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) 2016 Annual Report*.

Government of Canada. (2018b). *Notice to Stakeholders: Update on Collaborative efforts to promote the prudent use of Medically-Important Antimicrobials in animals*. http://www.hc-sc.gc.ca/dhp-mps/vet/antimicrob/amr-notice-ram-avis-2017-eng.php

Government of Canada. (2018c). *Notice to Stakeholders: Update on Collaborative efforts to promote the prudent use of Medically-Important Antimicrobials in animals*. http://www.hc-sc.gc.ca/dhp-mps/vet/antimicrob/amr-notice-ram-avis-2017-eng.php

Government of Canada. (2019). *Guidance document on classification of veterinary drugs and livestock feeds*. 2019-02-27. https://www.canada.ca/en/health-canada/services/drugs-health-products/veterinary-drugs/legislation-guidelines/guidance-documents/guidance-document-classification-veterinary-drugs-livestock-feeds/document.html

Grilli, E., Messina, M., Catelli, E., Morlacchini, M., & Piva, A. (2009). Pediocin a improves growth performance of broilers challenged with Clostridium perfringens. *Poultry Science*, *88*(10), 2152–2158. https://doi.org/10.3382/ps.2009-00160

Gulland, A. (2019). Countries still using antibiotics to fatten animals despite ban. *The Telegraph*.

Hammami, R., Fernandez, B., Lacroix, C., & Fliss, I. (2013). Anti-infective properties of bacteriocins: An update. *Cellular and Molecular Life Sciences*, *70*(16), 2947–2967. https://doi.org/10.1007/s00018-012-1202-3

Hammami, R., Fliss, I., & Corsetti, A. (2019). Editorial: Application of Protective Cultures and Bacteriocins for Food Biopreservation. *Frontiers in Microbiology*, *10*, 1561. https://doi.org/10.3389/fmicb.2019.01561

Hammami, R., Zouhir, A., Hamida, J. B., Neffati, M., Vergoten, G., Naghmouchi, K., & Fliss, I. (2009). Antimicrobial properties of aqueous extracts from three medicinal plants growing wild in arid regions of Tunisia. *Pharmaceutical Biology*, *47*(5), 452–457. https://doi.org/10.1080/13880200902822604

Hammami, R., Zouhir, A., Le Lay, C., Ben Hamida, J., & Fliss, I. (2010a). BACTIBASE second release: A database and tool platform for bacteriocin characterization. *BMC Microbiology*, *10*(1), 22. https://doi.org/10.1186/1471-2180-10-22

Hammami, R., Zouhir, A., Le Lay, C., Ben Hamida, J., & Fliss, I. (2010b). BACTIBASE second release: A database and tool platform for bacteriocin characterization. *BMC Microbiology*, *10*(1), 22. https://doi.org/10.1186/1471-2180-10-22

Han, S., Shin, M., Park, H., Kim, S., & Lee, W. (2014). Screening of Bacteriocin-producing Enterococcus faecalis Strains for Antagonistic Activities against Clostridium perfringens. *Korean Journal for Food Science of Animal Resources*, *34*(5), 614–621. https://doi.org/10.5851/kosfa.2014.34.5.614

Hanchi, H., Hammami, R., Kourda, R., Hamida, J. B., & Fliss, I. (2014). Bacteriocinogenic properties and in vitro probiotic potential of enterococci from Tunisian dairy products. *Archives of Microbiology*, *196*(5), 331–344. https://doi.org/10.1007/s00203-014-0978-y

Hanchi, H., Mottawea, W., Sebei, K., & Hammami, R. (2018). The Genus Enterococcus: Between Probiotic Potential and Safety Concerns—An Update. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.01791

Hofacre, C. L., Smith, J. A., & Mathis, G. F. (2018). An optimist's view on limiting necrotic enteritis and maintaining broiler gut health and performance in today's marketing, food safety, and regulatory climate. *Poultry Science*, *97*(6), 1929–1933. https://doi.org/10.3382/ps/pey082

Holmes, A. H., Moore, L. S. P., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., Guerin, P. J., & Piddock, L. J. V. (2016). Understanding the mechanisms and drivers of antimicrobial resistance. In *The Lancet* (Vol. 387, Issue 10014, pp. 176–187). https://doi.org/10.1016/S0140-6736(15)00473-0

Immerseel, F. V., Buck, J. D., Pasmans, F., Huyghebaert, G., Haesebrouck, F., & Ducatelle, R. (2004). Clostridium perfringens in poultry: An emerging threat for animal and public health. *Avian Pathology*, *33*(6), 537–549. https://doi.org/10.1080/03079450400013162

Jin, L. Z., Ho, Y. W., Abdullah, N., & Jalaludin, S. (2000). Digestive and Bacterial Enzyme Activities in Broilers Fed Diets Supplemented with Lactobacillus Cultures. *Poultry Science*, *79*(6), 886–891. https://doi.org/10.1093/ps/79.6.886

Johnson, Renée. (2009). *Potential Trade Implications of Restrictions on Antimicrobial Use in Animal Production*. 21.

Johnson, Ryan. (2018). *EU bans prophylactic use of antibiotic farming*. https://thepoultrysite.com/news/2018/10/eu-bans-prophylactic-use-of-antibiotics-in-farming

Jozefiak, D, Sip, A., Rawski, M., Rutkowski, A., Kaczmarek, S., Hojberg, O., Jensen, B. B., & Engberg, R. M. (2011). British Poultry Science Dietary divercin modifies gastrointestinal microbiota and improves growth performance in broiler chickens Dietary divercin modifies gastrointestinal microbiota and improves growth performance in broiler chickens. *British Poultry Science*, *52*(4), 492–499. https://doi.org/10.1080/00071668.2011.602963

Józefiak, D., Sip, A., Rutkowski, A., Rawski, M., Kaczmarek, S., Wołuń-Cholewa, M., Engberg, R. M., & Højberg, O. (2012). Lyophilized Carnobacterium divergens AS7 bacteriocin preparation improves performance of broiler chickens challenged with Clostridium perfringens. *Poultry Science*, *91*(8), 1899–1907. https://doi.org/10.3382/ps.2012-02151

Józefiak, Damian, Kierończyk, B., Juśkiewicz, J., Zduńczyk, Z., Rawski, M., Długosz, J., Sip, A., & Højberg, O. (2013). Dietary nisin modulates the gastrointestinal microbial ecology and enhances growth performance of the broiler chickens. *PLoS ONE*, *8*(12), 1–11. https://doi.org/10.1371/journal.pone.0085347

Józefiak, Damian, & Sip, A. (2013). Bacteriocins in poultry nutrition – a review. *Annals of Animal Science*, *13*(3), 449–462. https://doi.org/10.2478/aoas-2013-0031

Jozefiak, Damian, Sip, A., Rawski, M., Rutkowski, A., Kaczmarek, S., Hojberg, O., Jensen, B. B., & Engberg, R. M. (2011). Dietary divercin modifies gastrointestinal microbiota and improves growth performance in broiler chickens. *British Poultry Science*, *52*(4), 492–499. https://doi.org/10.1080/00071668.2011.602963

Kabir, S. M. L. (2009). The Role of Probiotics in the Poultry Industry. *International Journal of Molecular Sciences*, *10*(8), 3531–3546. https://doi.org/10.3390/ijms10083531

Kaiser, A., Willer, T., Steinberg, P., & Rautenschlein, S. (2017). Establishment of an *In Vitro* Intestinal Epithelial Cell Culture Model of Avian Origin. *Avian Diseases*, *61*(2), 229–236. https://doi.org/10.1637/11524-110216-Reg.1

Kallapura, G. K., Hernandez-, X., Piekarski, A., Lassiter, K., Pumford, N. R., Tellez, G., Bottje, W. G., Hargis, B. M., & Faulkner, O. B. (2015). Development of an ex Vivo Ileal Explant Culture Method for Amplified Production and Differential Measurement of Nitrite. *International Journal of Poultry Science*, *14*(5), 245–251. https://doi.org/10.3923/ijps.2015.245.251

Kapoor, G., Saigal, S., & Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: A guide for clinicians. In *Journal of Anaesthesiology Clinical Pharmacology* (Vol. 33, Issue 3, pp. 300–305). Wolters Kluwer -- Medknow Publications. https://doi.org/10.4103/joacp.JOACP_349_15

Kieronczyk, B., Sassek, M., Pruszynska-Oszmalek, E., Kolodziejski, P., Rawski, M., Swiatkiewicz, S., & Józefiak, D. (2017). The physiological response of broiler chickens to the dietary supplementation of the bacteriocin nisin and ionophore coccidiostats. *Poultry Science*, *96*(11), 4026–4037. https://doi.org/10.3382/ps/pex234

Kim, J. Y., Young, J. A., Gunther, N. W., & Lee, J. L. (2015). Inhibition of salmonella by bacteriocin-producing lactic acid bacteria derived from U.S. kimchi and broiler chicken. *Journal of Food Safety*, *35*(1), 1–12. https://doi.org/10.1111/jfs.12141

Kim, S.-Y., Ogawa, Y., & Adachi, Y. (2006). Canine Intestinal Lactic Acid Bacteria Agglutinated with Concanavalin A. *Journal of Veterinary Medical Science*, *68*(12), 1351–1354. https://doi.org/10.1292/jvms.68.1351

Kizerwetter-Swida, M., & Binek, M. (2009). Protective effect of potentially probiotic Lactobacillus strain on infection with pathogenic bacteria in chickens. *Polish Journal of Veterinary Sciences*, *12*(1), 15–20.

Konkel, M. E., Christensen, J. E., Dhillon, A. S., Lane, A. B., Hare-Sanford, R., Schaberg, D. M., & Larson, C. L. (2007). Campylobacter jejuni Strains Compete for Colonization in Broiler Chicks. *Applied and Environmental Microbiology*, *73*(7), 2297–2305. https://doi.org/10.1128/AEM.02193-06

Kumar, S., Chen, C., Indugu, N., Werlang, G. O., Singh, M., Kim, W. K., & Thippareddi, H. (2018). Effect of antibiotic withdrawal in feed on chicken gut microbial dynamics, immunity, growth performance and prevalence of foodborne pathogens. *PLoS ONE*, *13*(2), 1–23. https://doi.org/10.1371/journal.pone.0192450

Lan, P. T. N., Binh, L. T., & Benno, Y. (2003). *Impact of two probiotic Lactobacillus strains feeding on fecal lactobacilli and weight gains in chicken*. *49*(1), 29–36. https://doi.org/10.2323/jgam.29.29

Larson, C. L., Shah, D. H., Dhillon, A. S., Call, D. R., Ahn, S., Haldorson, G. J., Davitt, C., & Konkel, M. E. (2008). Campylobacter jejuni invade chicken LMH cells inefficiently and stimulate differential expression of the chicken CXCLi1 and CXCLi2 cytokines. *Microbiology*, *154*(12), 3835–3847. https://doi.org/10.1099/mic.0.2008/021279-0

Lauková, A., Kandričáková, A., & Ščerbová, J. (2015). Use of bacteriocin-producing, probiotic strain *Enterococcus faecium* AL41 to control intestinal microbiota in farm ostriches. *Letters in Applied Microbiology*, *60*(6), 531–535. https://doi.org/10.1111/lam.12409

Laulund, S., Wind, A., Derkx, P., & Zuliani, V. (2017). Regulatory and Safety Requirements for Food Cultures. *Microorganisms*, *5*(2), 28. https://doi.org/10.3390/microorganisms5020028

Li, J., Hao, H., Cheng, G., Liu, C., Ahmed, S., Shabbir, M. A. B. B., Hussain, H. I., Dai, M., & Yuan, Z. (2017). Microbial shifts in the intestinal microbiota of Salmonella infected chickens in response to enrofloxacin. *Frontiers in Microbiology*, *8*(SEP), 1–14. https://doi.org/10.3389/fmicb.2017.01711

Li, Q., Li, Y., Xia, J., Wang, X., Yin, K., Hu, Y., Yin, C., Liu, Z., & Jiao, X. (2019). Virulence of Salmonella entericaserovar Pullorum isolates compared using cell-based and chicken embryo infection models. *Poultry Science*, *98*(3), 1488–1493. https://doi.org/10.3382/ps/pey482

Lin, J., Sahin, O., Michel, L. O., & Zhang, Q. (2003). Critical Role of Multidrug Efflux Pump CmeABC in Bile Resistance and In Vivo Colonization of Campylobacter jejuni. *Infection and Immunity*, *71*(8), 4250–4259. https://doi.org/10.1128/IAI.71.8.4250-4259.2003

Liu, L., Lin, L., Zheng, L., Tang, H., Fan, X., Xue, N., Li, M., Liu, M., & Li, X. (2018). Cecal microbiome profile altered by Salmonella enterica, serovar Enteritidis inoculation in chicken. *Gut Pathogens*, *10*(1), 34. https://doi.org/10.1186/s13099-018-0261-x

Lo Verso, L., Lessard, M., Talbot, G., Fernandez, B., & Fliss, I. (2018). Isolation and Selection of Potential Probiotic Bacteria from the Pig Gastrointestinal Tract. *Probiotics and Antimicrobial Proteins*, *10*(2), 299–312. https://doi.org/10.1007/s12602-017-9309-3

Ludlow, K. (2010). Australian Pesticides and Veterinary Medicines Authority. In D. Guston, *Encyclopedia of Nanoscience and Society*. SAGE Publications, Inc. https://doi.org/10.4135/9781412972093.n22

Maki, J. J., Klima, C. L., Sylte, M. J., & Looft, T. (2019). The Microbial Pecking Order: Utilization of Intestinal Microbiota for Poultry Health. *Microorganisms*, *7*(10), 376. https://doi.org/10.3390/microorganisms7100376

Maron, D. F., Smith, T. J., & Nachman, K. E. (2013). *Restrictions on antimicrobial use in food animal production: An international regulatory and economic survey*. https://doi.org/10.1186/1744-8603-9-48

Marshall, B. M., & Levy, S. B. (2011a). Food animals and antimicrobials: Impacts on human health. *Clinical Microbiology Reviews*, *24*(4), 718–733. https://doi.org/10.1128/CMR.00002-11

Marshall, B. M., & Levy, S. B. (2011b). Food animals and antimicrobials: Impacts on human health. *Clinical Microbiology Reviews*, *24*(4), 718–733. https://doi.org/10.1128/CMR.00002-11

Martin-Platero, A. M., Valdivia, E., Ruiz-Rodriguez, M., Soler, J. J., MArtin-Vivaldi, M., Maqueda, M., & Martinez-Bueno, M. (2006). Characterization of Antimicrobial Substances Produced by Enterococcus faecalis MRR 10-3, Isolated from the Uropygial Gland of the Hoopoe (Upupa epops). *Applied and Environmental Microbiology*, *72*(6), 4245–4249. https://doi.org/10.1128/AEM.02940-05

Messaoudi, S., Manai, M., Kergourlay, G., Prévost, H., Connil, N., Chobert, J.-M., & Dousset, X. (2013a). Lactobacillus salivarius: Bacteriocin and probiotic activity. *Food Microbiology*, *36*(2), 296–304. https://doi.org/10.1016/j.fm.2013.05.010

Messaoudi, S., Manai, M., Kergourlay, G., Prévost, H., Connil, N., Chobert, J.-M., & Dousset, X. (2013b). Lactobacillus salivarius: Bacteriocin and probiotic activity. *Food Microbiology*, *36*(2), 296–304. https://doi.org/10.1016/j.fm.2013.05.010

Messaoudi, Soumaya, Kergourlay, G., Rossero, A., Ferchichi, M., Prévost, H., Drider, D., Manai, M., & Dousset, X. (2011). Identification of lactobacilli residing in chicken ceca with antagonism against Campylobacter. *International Microbiology*, *14*(2), 103–110. https://doi.org/10.2436/20.1501.01.140

Micciche, A. C., Foley, S. L., Pavlidis, H. O., McIntyre, D. R., & Ricke, S. C. (2018a). A review of prebiotics against Salmonella in poultry: Current and future potential for microbiome research applications. *Frontiers in Veterinary Science*, *5*(AUG), 1–11. https://doi.org/10.3389/fvets.2018.00191

Micciche, A. C., Foley, S. L., Pavlidis, H. O., McIntyre, D. R., & Ricke, S. C. (2018b). A Review of Prebiotics Against Salmonella in Poultry: Current and Future Potential for Microbiome Research Applications. *Frontiers in Veterinary Science*. https://doi.org/10.3389/fvets.2018.00191

Micciche, A. C., Foley, S. L., Pavlidis, H. O., McIntyre, D. R., & Ricke, S. C. (2018c). A Review of Prebiotics Against Salmonella in Poultry: Current and Future Potential for Microbiome Research Applications. *Frontiers in Veterinary Science*, *5*, 191. https://doi.org/10.3389/fvets.2018.00191

Mohanty, D., Panda, S., Kumar, S., & Ray, P. (2019). In vitro evaluation of adherence and anti-infective property of probiotic Lactobacillus plantarum DM 69 against Salmonella enterica. *Microbial Pathogenesis*, *126*(November), 212–217. https://doi.org/10.1016/j.micpath.2018.11.014

Mottet, A., & Tempio, G. (2017a). Global poultry production: Current state and future outlook and challenges. *World's Poultry Science Journal*, *73*(2), 245–256. https://doi.org/10.1017/S0043933917000071

Mottet, A., & Tempio, G. (2017b). Global poultry production: Current state and future outlook and challenges. *World's Poultry Science Journal*, *73*(2), 245–256. https://doi.org/10.1017/S0043933917000071

Mukai, T., Arihara, K., Ikeda, A., Nomura, K., Suzuki, F., & Ohori, H. (2003). Lactobacillus kitasatonis sp. Nov., from chicken intestine. *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY*, *53*(6), 2055–2059. https://doi.org/10.1099/ijs.0.02815-0

Nilsen, T., Nes, I. F., & Holo, H. (2003). Enterolysin A, a Cell Wall-Degrading Bacteriocin from Enterococcus faecalis LMG 2333. *Applied and Environmental Microbiology*, *69*(5), 2975–2984. https://doi.org/10.1128/AEM.69.5.2975-2984.2003

Oakley, B. B., Lillehoj, H. S., Kogut, M. H., Kim, W. K., Maurer, J. J., Pedroso, A., Lee, M. D., Collett, S. R., Johnson, T. J., & Cox, N. A. (2014a). The chicken gastrointestinal microbiome. *FEMS Microbiology Letters*, *360*(2), 100–112. https://doi.org/10.1111/1574-6968.12608

Oakley, B. B., Lillehoj, H. S., Kogut, M. H., Kim, W. K., Maurer, J. J., Pedroso, A., Lee, M. D., Collett, S. R., Johnson, T. J., & Cox, N. A. (2014b). The chicken gastrointestinal microbiome. *FEMS Microbiology Letters*, *360*(2), 100–112. https://doi.org/10.1111/1574-6968.12608

Oladeinde, A., Cook, K., Lakin, S. M., Woyda, R., Abdo, Z., Looft, T., Herrington, K., Zock, G., Lawrence, J. P., Thomas, J. C., Beaudry, M. S., & Glenn, T. (2019). Horizontal Gene

Transfer and Acquired Antibiotic Resistance in *Salmonella enterica* Serovar Heidelberg following *In Vitro* Incubation in Broiler Ceca. *Applied and Environmental Microbiology*, *85*(22), e01903-19, /aem/85/22/AEM.01903-19.atom. https://doi.org/10.1128/AEM.01903-19

O'Neill, J. (2014). Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations. In *Review on Antimicrobial Resistance* (Issue December). https://doi.org/10.1038/510015a

Ongey, E. L., & Neubauer, P. (2016). Lanthipeptides: Chemical synthesis versus in vivo biosynthesis as tools for pharmaceutical production. *Microbial Cell Factories*, *15*(1), 97. https://doi.org/10.1186/s12934-016-0502-y

O'Toole, P. W., & Cooney, J. C. (2008). Probiotic Bacteria Influence the Composition and Function of the Intestinal Microbiota. *Interdisciplinary Perspectives on Infectious Diseases*, *2008*, 1–9. https://doi.org/10.1155/2008/175285

Pan, D., & Yu, Z. (2013a). Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes*. https://doi.org/10.4161/gmic.26945

Pan, D., & Yu, Z. (2013b). Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes*, *5*(1), 108–119. https://doi.org/10.4161/gmic.26945

Pan, D., & Yu, Z. (2014). Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes*, *5*(1), 108–119. https://doi.org/10.4161/gmic.26945

Park, Y. H., Hamidon, F., Rajangan, C., Soh, K. P., Gan, C. Y., Lim, T. S., Abdullah, W. N. W., & Liong, M. T. (2016). Application of Probiotics for the Production of Safe and High-quality Poultry Meat. *Korean Journal for Food Science of Animal Resources*, *36*(5), 567–576. https://doi.org/10.5851/kosfa.2016.36.5.567

Patterson, C. A. (2008). *Probiotics Benefits beyond basic nutrition*.

Prescott, J. F. (2008). Antimicrobial use in food and companion animals. *Animal Health Research Reviews / Conference of Research Workers in Animal Diseases*, *9*(2), 127–133. https://doi.org/10.1017/S1466252308001473

Priyodip, P., & Balaji, S. (2019). An in vitro chicken gut model for the assessment of phytase producing bacteria. *3 Biotech*, *9*(8), 294. https://doi.org/10.1007/s13205-019-1825-2

Qiao, J., Zhang, Q., Alali, W. Q., Wang, J., Meng, L., Xiao, Y., Yang, H., Chen, S., Cui, S., & Yang, B. (2017). Characterization of extended-spectrum β-lactamases (ESBLs)-producing Salmonella in retail raw chicken carcasses. *International Journal of Food Microbiology*, *248*, 72–81. https://doi.org/10.1016/j.ijfoodmicro.2017.02.016

Ralte, R. (2018). *Antibiotic use in poultry feeds to be phased out from 2018*. The Poultry Site. https://thepoultrysite.com/news/2018/01/antibiotic-use-in-poultry-feed-to-be-phased-out-from-2018

Rath, N. C., Liyanage, R., Gupta, A., Packialakshmi, B., & Lay, J. O. (2018). A method to culture chicken enterocytes and their characterization. *Poultry Science*, *97*(11), 4040–4047. https://doi.org/10.3382/ps/pey248

Redondo, L. M., Chacana, P. A., Dominguez, J. E., & Fernandez Miyakawa, M. E. (2014). Perspectives in the use of tannins as alternative to antimicrobial growth promoter factors in poultry. *Frontiers in Microbiology*, *5*. https://doi.org/10.3389/fmicb.2014.00118

Rychen, G., Aquilina, G., Azimonti, G., Bampidis, V., Bastos, M. de L., Bories, G., Chesson, A., Cocconcelli, P. S., Flachowsky, G., Gropp, J., Kolar, B., Kouba, M., López-Alonso, M., López Puente, S., Mantovani, A., Mayo, B., Ramos, F., Saarela, M., Villa, R. E., … Galobart, J. (2018a). Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA Journal*, *16*(3), 1–24. https://doi.org/10.2903/j.efsa.2018.5206

Rychen, G., Aquilina, G., Azimonti, G., Bampidis, V., Bastos, M. de L., Bories, G., Chesson, A., Cocconcelli, P. S., Flachowsky, G., Gropp, J., Kolar, B., Kouba, M., López-Alonso, M., López Puente, S., Mantovani, A., Mayo, B., Ramos, F., Saarela, M., Villa, R. E., … Galobart, J. (2018b). Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA Journal*, *16*(3), 1–24. https://doi.org/10.2903/j.efsa.2018.5206

Rychen, G., Aquilina, G., Azimonti, G., Bampidis, V., Bastos, M. de L., Bories, G., Chesson, A., Cocconcelli, P. S., Flachowsky, G., Gropp, J., Kolar, B., Kouba, M., López-Alonso, M., López Puente, S., Mantovani, A., Mayo, B., Ramos, F., Saarela, M., Villa, R. E., … Martino, L. (2017). Guidance on the assessment of the safety of feed additives for the target species. *EFSA Journal*, *15*(10), 5021. https://doi.org/10.2903/j.efsa.2017.5021

Sakpuaram, K., Wajjwalku, T., Nitisinprasert, W., Swetwiwathana, S., Zendo, A., Fujita, T., Nakayama, K., & Sonomoto, J. (2006). Faculty of Agricultural Industry, King Mongkut's Institute of Technology Ladkrabang (KMITL). In *Ph.D.(Agricultural Science), Asst. Prof* (Issue 5).

Santin, E., Hayashi, R. M., Wammes, J. C., Gonzalez-Esquerra, R., Carazzolle, M. F., Freire, C. C. de M., Monzani, P. S., & Cunha, A. F. da. (2017). Phenotypic and Genotypic Features of a Salmonella Heidelberg Strain Isolated in Broilers in Brazil and Their Possible Association to Antibiotics and Short-Chain Organic Acids Resistance and Susceptibility. *Frontiers in Veterinary Science*. https://doi.org/10.3389/fvets.2017.00184

Silbergeld, E. K., Graham, J., & Price, L. B. (2008). Industrial food animal production, antimicrobial resistance, and human health. *Annu. Rev. Public Health*, *29*, 151–169. https://doi.org/10.1146/annurev.publhealth.29.020907.090904

Snell, B. (2019). *Analysis of AMR Plans in the Western Pacific and South-East Asia Regions TWN Third World Network*.

Spivey, M. A., Dunn-Horrocks, S. L., & Duong, T. (2014). Epithelial cell adhesion and gastrointestinal colonization of Lactobacillus in poultry. *Poultry Science*, *93*(11), 2910–2919. https://doi.org/10.3382/ps.2014-04076

Sri Lanka. (2017). *National Strategic Plan for Combating Antimicrobial Resistance in Sri Lanka*.

Stern, N. J., Svetoch, E. A., Eruslanov, B. V., Perelygin, V. V., Mitsevich, E. V., Mitsevich, I. P., Pokhilenko, V. D., Levchuk, V. P., Svetoch, O. E., & Seal, B. S. (2006). Isolation of a Lactobacillus salivarius strain and purification of its bacteriocin, which is inhibitory to Campylobacter jejuni in the chicken gastrointestinal system. *Antimicrobial Agents and Chemotherapy*, *50*(9), 3111–3116. https://doi.org/10.1128/AAC.00259-06

Suresh, G., Das, R. K., Kaur Brar, S., Rouissi, T., Avalos Ramirez, A., Chorfi, Y., & Godbout, S. (2018). Alternatives to antibiotics in poultry feed: Molecular perspectives. In *Critical Reviews in Microbiology*. https://doi.org/10.1080/1040841X.2017.1373062

Svetoch, E. A., & Stern, N. J. (2010). Bacteriocins to control Campylobacter spp. In poultry-A review. *Poultry Science*, *89*(8), 1763–1768. https://doi.org/10.3382/ps.2010-00659

Svetoch, Edward A., Eruslanov, B. V., Levchuk, V. P., Perelygin, V. V., Mitsevich, E. V., Mitsevich, I. P., Stepanshin, J., Dyatlov, I., Seal, B. S., & Stern, N. J. (2011). Isolation of Lactobacillus salivarius 1077 (NRRL B-50053) and characterization of its bacteriocin, including the antimicrobial activity spectrum. *Applied and Environmental Microbiology*, *77*(8), 2749–2754. https://doi.org/10.1128/AEM.02481-10

The Chicken Farmers. (2016). *New Poultry Code of Practice is Complete!*

Thomas, M. K., Murray, R., Flockhart, L., Pintar, K., Pollari, F., Fazil, A., Nesbitt, A., & Marshall, B. (2013). Estimates of the Burden of Foodborne Illness in Canada for 30 Specified Pathogens and Unspecified Agents, Circa 2006. *Foodborne Pathogens and Disease*, *10*(7), 639–648. https://doi.org/10.1089/fpd.2012.1389

Timbermont, L., De Smet, L., Van Nieuwerburgh, F., Parreira, V. R., Van Driessche, G., Haesebrouck, F., Ducatelle, R., Prescott, J., Deforce, D., Devreese, B., & Van Immerseel, F. (2014). Perfrin, a novel bacteriocin associated with netB positive Clostridium perfringens strains from broilers with necrotic enteritis. *Veterinary Research*, *45*(1), 40. https://doi.org/10.1186/1297-9716-45-40

Trung, N. V., Matamoros, S., Carrique-Mas, J. J., Nghia, N. H., Nhung, N. T., Chieu, T. T. B., Mai, H. H., van Rooijen, W., Campbell, J., Wagenaar, J. A., Hardon, A., Mai, N. T. N., Hieu, T. Q., Thwaites, G., de Jong, M. D., Schultsz, C., & Hoa, N. T. (2017). Zoonotic transmission of mcr-1 colistin resistance gene from small-scale poultry farms, Vietnam. *Emerging Infectious Diseases*. https://doi.org/10.3201/eid2303.161553

Turner, S., Pryer, K. M., Miao, V. P. W., & Palmer, J. D. (1999). Investigating Deep Phylogenetic Relationships among Cyanobacteria and Plastids by Small Subunit rRNA Sequence Analysis. *The Journal of Eukaryotic Microbiology*, *46*(4), 327–338. https://doi.org/10.1111/j.1550-7408.1999.tb04612.x

Van Immerseel, F. (2003). Invasion of Salmonella enteritidis in avian intestinal epithelial cells in vitro is influenced by short-chain fatty acids. *International Journal of Food Microbiology*, *85*(3), 237–248. https://doi.org/10.1016/S0168-1605(02)00542-1

Velge, P. (2002). Establishment and characterization of partially differentiated chicken enterocyte cell clones. *European Journal of Cell Biology*, *81*(4), 203–212. https://doi.org/10.1078/0171-9335-00237

Verraes, C., Van Boxstael, S., Van Meervenne, E., Coillie, E. Van, Butaye, P., Catry, B., De Schaetzen, M.-A., Van Huffel, X., Imberechts, H., Dierick, K., Daube, G., Saegerman, C., De Block, J., Dewulf, J., & Herman, L. (2013). Antimicrobial Resistance in the Food Chain: A Review. *Int. J. Environ. Res. Public Health*, *10*, 10. https://doi.org/10.3390/ijerph10072643

Vieco-Saiz, N., Belguesmia, Y., Raspoet, R., Auclair, E., Gancel, F., Kempf, I., & Drider, D. (2019). Benefits and Inputs From Lactic Acid Bacteria and Their Bacteriocins as Alternatives to Antibiotic Growth Promoters During Food-Animal Production. *Frontiers in Microbiology*, *10*(February), 1–17. https://doi.org/10.3389/fmicb.2019.00057

Vriezen, J. A. C., Valliere, M., & Riley, M. A. (2009). The Evolution of Reduced Microbial Killing. *Genome Biology and Evolution*, *1*, 400–408. https://doi.org/10.1093/gbe/evp042

Walia, K., Sharma, M., Vijay, S., & Shome, B. R. (2019). Understanding policy dilemmas around antibiotic use in food animals & offering potential solutions. *The Indian Journal of Medical Research*, *149*(2), 107–118. https://doi.org/10.4103/ijmr.IJMR_2_18

Wan, M. L., Forsythe, S. J., & El-Nezami, H. (2018). Probiotics interaction with foodborne pathogens: A potential alternative to antibiotics and future challenges. *Critical Reviews in Food Science and Nutrition*, 1–29. https://doi.org/10.1080/10408398.2018.1490885

Wan, M. L. L. Y., Forsythe, S. J., & El-Nezami, H. (2018). Probiotics interaction with foodborne pathogens: A potential alternative to antibiotics and future challenges. *Critical Reviews in Food Science and Nutrition*, *July*, 1–14. https://doi.org/10.1080/10408398.2018.1490885

Wang, H. T., Li, Y. H., Chou, I. P., Hsieh, Y. H., Chen, B. J., & Chen, C. Y. (2013). Albusin B modulates lipid metabolism and increases antioxidant defense in broiler chickens by a proteomic approach. *Journal of the Science of Food and Agriculture*, *93*(2), 284–292. https://doi.org/10.1002/jsfa.5754

Wang, H. T., Yu, C., Hsieh, Y. H., Chen, S. W., Chen, B. J., & Chen, C. Y. (2011). Effects of albusin B (a bacteriocin) of Ruminococcus albus 7 expressed by yeast on growth performance and intestinal absorption of broiler chickens-its potential role as an alternative to feed antibiotics. *Journal of the Science of Food and Agriculture*, *91*(13), 2338–2343. https://doi.org/10.1002/jsfa.4463

Wang, Q., Gong, J., Huang, X., Yu, H., & Xue, F. (2009). In vitro evaluation of the activity of microencapsulated carvacrol against Escherichia coli with K88 pili. *Journal of Applied Microbiology*, *107*(6), 1781–1788. https://doi.org/10.1111/j.1365-2672.2009.04374.x

Wang, Y., Hu, Y., Cao, J., Bi, Y., Lv, N., Liu, F., Liang, S., Shi, Y., Jiao, X., Gao, G. F., & Zhu, B. (2019). Antibiotic resistance gene reservoir in live poultry markets. *Journal of Infection*, *78*(6), 445–453. https://doi.org/10.1016/j.jinf.2019.03.012

WHO. (2017). *Namibia's ban on antibitocis in healthy animals drives meat exports*. https://www.who.int/news-room/feature-stories/detail/namibia-s-ban-on-antibiotics-in-healthy-animals-drives-meat-exports

Wu, Z. (2019). *Antibiotic use and antibiotic resistance in food-producing animals in China* (No. 134; OECD Food, Agriculture and Fisheries Papers). https://doi.org/10.1787/4adba8c1-en

Yamazaki, M., Ohtsu, H., Yakabe, Y., Kishima, M., & Abe, H. (2012). In vitro screening of lactobacilli isolated from chicken excreta to control Salmonella Enteritidis and Typhimurium. *British Poultry Science*, *53*(2), 183–189. https://doi.org/10.1080/00071668.2012.678814

Yang, H., Paruch, L., Chen, X., van Eerde, A., Skomedal, H., Wang, Y., Liu, D., & Liu Clarke, J. (2019). Antibiotic Application and Resistance in Swine Production in China: Current Situation and Future Perspectives. *Frontiers in Veterinary Science*, *6*. https://doi.org/10.3389/fvets.2019.00136

Yang, X., Brisbin, J., Yu, H., Wang, Q., Yin, F., Zhang, Y., Sabour, P., Sharif, S., & Gong, J. (2014). Selected Lactic Acid-Producing Bacterial Isolates with the Capacity to Reduce Salmonella Translocation and Virulence Gene Expression in Chickens. *PLoS ONE*, *9*(4), e93022. https://doi.org/10.1371/journal.pone.0093022

Yuan, C., He, Q., Li, J., Azzam, M. M., Lu, J., & Zou, X. (2015). Evaluation of embryonic age and the effects of different proteases on the isolation and primary culture of chicken intestinal epithelial cells *in vitro*: Primary Culture of Chicken IEC *In Vitro*. *Animal Science Journal*, *86*(6), 588–594. https://doi.org/10.1111/asj.12337

Zhu, Y., Lai, H., Zou, L., Yin, S., Wang, C., Han, X., Xia, X., Hu, K., He, L., Zhou, K., Chen, S., Ao, X., & Liu, S. (2017). Antimicrobial resistance and resistance genes in Salmonella strains isolated from broiler chickens along the slaughtering process in China.

International Journal of Food Microbiology.

https://doi.org/10.1016/j.ijfoodmicro.2017.07.023