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SCREENING AND PROBIOTIC CHARACTERIZATION OF BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA ISOLATED FROM BROILERS AND KIMCHI

by

Janay A. Young

A THESIS

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To my mother and my father

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Abstract

Lactic acid bacteria (LAB), are generally recognized as safe (GRAS) for use in food products. They are able to inhibit the growth of pathogens; such as, *Salmonella,* therefore they are commonly used as probiotics. LAB are found in Korean Kimchi; a traditional dish made from vegetables then stored for fermentation. In this study, LAB were isolated from kimchi and from broiler chicken intestines and feces. 388 isolates were screened for their ability to inhibit the growth of *Salmonella* Typhimurium, *S.* Newport, *S.* Heidelberg, and *S.* Enteritidis by the agar well diffusion method. Isolates with positive results were then screened further for the production of bacteriocins. For this screening, the agar well diffusion method was also used; Cell-free supernatants were treated with sodium hydroxide and catalase and used as "crude bacteriocins". There were a total of 50 isolates which showed positive bacteriocin-production by zones of inhibition against 1 or more *Salmonella* species.

The probiotic abilities of these candidates were then studied by bile and acid resistance, antibiotic susceptibility, and enzyme tolerance. All isolates were able to resist a 0.3% bile salt solution except com-35 and F-34 isolated from commercial kimchi and broiler fecal materials, respectively. The acid tolerance of the isolates varied; some LAB strains were completely killed when exposed to a gastric solution for 2 h while others were able to maintain viable cells up to Log 6 CFU/ml. The isolates with acid and bile

resistance were exposed to alpha-amylase and lysozyme solutions to assess their abilities to successfully travel through the conditions of the gastrointestinal tract. Optical densities were measured at 0 h, 4 h, and 24 h. A total of 16 surviving isolates were then enumerated by plate count method. Antibiotic susceptibility of the isolates was tested by the agar well diffusion method. Among the isolates showing enzyme tolerance, all showed resistance to the antibiotics kanamycin and streptomycin, with com-54, com-73, and com-75 being exceptions; those three isolates were inhibited by all six tested antibiotics.

By 16S rDNA sequencing 16 isolates were identified; among them, a total of 5 LAB were selected as final candidates for probiotic use and *Salmonella* inhibition. By using the BLAST system on GenBank, the isolates Cab-18, Cuc-1, Com-54, F-6, and F-59 have been identified as *Lactobacillus casei, Lactobacillus saniviri, Leuconostoc mesenteroides, Lactobacillus crispatus, Lactobacillus johnsonii*, respectively.

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Literature Review

Lactic Acid Bacteria

Lactic acid bacteria (LAB) are gram positive, non spore forming, non motile, and rod or coccus shaped. LAB have been widely studied and used in the food industry because of their ability to decrease pH by producing lactic acid; however, they are also known to produce other compounds such as hydrogen peroxide, carbon dioxide, ethanol, flavor compounds, and bacteriocins (Lee *et al.*, 1992; Cheigh *et al.*, 1994; Nes *et al.*, 1996; Yun *et al.*, 1996; Oyetayo *et al.*, 2003). The genera of bacteria included as lactic acid bacteria, which are commonly associated with fermented foods, include *Carnobacterium, Enterococcus, Lactococcus, Lactobacillus, Leuconostoc, Oenococcus, Pediococci Streptococcus, Tetragenococcus, Vagococcus, and Weisella* (Stiles and Holzapfel, 1997; Nes *et al.*, 2007).

The interest in LAB began with the research of Elie Metchnikoff. Metchnikoff made observations in his research that the presence of certain bacteria in the intestines, inhibited growth of pathogens and prevented infections in the host. He noticed that Bulgarians, who drank dairy products fermented with *Lactobacillus*, lived longer and better lives and thus he began his research with *Lactobacillus* strains (Bibiel, 1988). Since then, LAB have been commonly studied and isolated from different fermented foods. LAB are important for the fermentation process of Sauerkraut, which is an

American fermented food dish, and Kimchi, a Korean fermented food dish. Once consumed, these LAB are known to inhabit the gastrointestinal tract of the host and have been commonly used as probiotics to inhibit the growth of pathogens (Mead, 2000). Pathogen inhibition can be attributed to the ability of LAB to produce lactic acid and acetic acid which lower the pH of their environment. This is a major contributing characteristic to their ability to hinder or eliminate normal functions of pathogenic bacteria (Shah, 2007).

Bacteriocins

Bacteriocins are peptides that are capable of inhibiting the growth of other microorganisms including pathogenic and food spoilage bacteria (Maria, 2012). These peptides can have varying size, structures, and functions. There are four established classes of bacteriocins with classes I and II being the most commonly studied (Nes, 1996). Class I, called lantibiotics, consist of bacteriocins that contain the amino acid lanthionine. Members of this class are the smallest in size (less than 5 kDa). There are two subgroups for class I. Subgroup A bacteriocins are long rod shaped and have a positive charge while subgroup B are circular shaped bacteriocins but have no charge. The most commonly studied bacteriocin, Nisin, is produced by *Lactobacillus lactis* and belongs to class I subgroup A. It is the only commercialized bacteriocin available to date.

Class II bacteriocins are much simpler than class I; they do not contain lanthionine and are less than 10 kDa in size. This class of bacteriocins are "non-modified", "heat stable" (Nes, 2007) and can be divided into 5 subgroups. Class IIa show antimicrobial activity against *Listeria*, IIb requires two peptides for proper function, IIc consists of bacteriocins that do not fit into other subgroups, IId are "leaderless" meaning that they can be produced by many different bacteria, and IIe which are bacteriocins produced by other proteins that have been broken down (Nes *et al.*, 2007). Class III bacteriocins are "heat-labile" and much larger than the previously mentioned classes (larger than 30 kDa). Lastly, class IV bacteriocins are the most complex and can contain lipids and/or carbohydrates (Stern *et al.*, 2006); they are referred to as cyclic bacteriocins.

Interestingly, it is common for some lactic acid bacteria to produce more than one bacteriocin in addition to the other antimicrobial compounds they produce. Furthermore, the bacteriocins produced might actually have varying functions and/or characteristics and belong to completely different characterized classes. For example, one strain of *E. faecium* produces enterocin A and enterocin B, which belong to separate classes. This has also been shown in a laboratory research study by Eijsink *et al.*, where, during the purification process, some of the inhibitory properties of the bacteriocins were lost. This was due to other bacteriocin-like substances that were naturally present, being separated and removed during purification from the characterized bacteriocin (Eijsink *et al.*, 1998).

This showed that more than one bacteriocin had been produced and that the functions of those bacteriocins was not the same.

Bacteriocins have an interesting range of bacteria that they normally show activity against. For example, a bacteriocin produced by a gram-negative bacteria will usually show antimicrobial activity against other gram-negative bacteria that are very closely-related to its producing strain; On the other hand, a bacteriocin produced by a gram-positive bacteria will usually show activity against a larger range of gram-positive bacteria and can also show action towards gram-negative bacteria (Tagg *et al.*, 1976; Balciunas, 2012). An important factor in a bacteriocins ability to inhibit a specific pathogen, is whether or not that bacteriocin undergoes modifications and/or purifications. Bacteriocins in class I go through "post-translational modifications" but those in class II do not which affects their function towards pathogenic bacteria (Nes *et al.*, 2007).

The antimicrobial function of bacteriocins comes mainly from their interaction with the cytoplasmic membrane of pathogenic bacteria. This function and interaction can be disrupted by certain compounds commonly found in food; however, the disruption can be countered by EDTA (Ganzle *et al.*, 1999). EDTA compromises the outer membrane of bacteria allowing for easier access and penetration by bacteriocins. EDTA and bacteriocins have demonstrated that the effect of the two compounds when combined significantly reduced the amount of *Salmonella* and *E. coli* on broiler carcasses (Shefet *et*

al., 1995). In addition to EDTA, NaCl and low pH also have a positive effect of bacteriocin activity (Ganzle *et al.*, 1999).

The effect of specificity of each bacteriocin towards target organisms depends heavily on the presence of additional disulfide bonds; bacteriocins that have been reduced are substantially less effective against pathogens. It is interesting to note that bacteriocin function and antimicrobial effect has been proven to vary drastically depending on which indicator strain is used. Sakacin P, for example, can be only slightly active against one bacteria yet have a strong effect on others (Eijsink *et al.*, 1998). Research by Diop *et al.*, showed that *Lactococcus lactis* subsp. *lactis* produced bacteriocins that showed strong antimicrobial activity against *Listeria monocytogenes* and *Bacillus cereus* (2007) and another species, while *Lactobacillus salivarius*, produces a bacteriocin that is active against *Campylobacter jejuni* (Stern *et al.*, 2006). One study suggests that the acidic environment in the stomach actually helps the function of LAB by assisting the bacteriocins with cell destruction (Ganzle *et al.*, 1999). It is the production of these bacteriocins and acid compounds that puts LAB ahead of other bacteria for use as probiotics (Marteau *et al.*, 1993; Salminem *et al.*, 1998).

Probiotics

Lactic acid bacteria (LAB) are often used as probiotics and are generally recognized as safe (GRAS) which ensures that they can be safely added to food for human consumption; LAB are proven safe because they are naturally found in foods (Nes *et al.*, 2007). Probiotics are live microorganisms that offer some benefit to their host; usually by enhancing the micro flora of the intestines (Fuller, 1989). Different species of LAB have the ability to slow and/or inhibit the growth of pathogenic bacteria such as *Salmonella* and *Campylobacter*; *Streptococcus salivarius* and *Enterococcus faecalis* have been isolated, studied and proven to have antagonistic activity against such pathogens (Hwanhlem *et al.*, 2010).

The health benefits of LAB include, but are not limited to the following: prevention of infections, immune system aid, decreased allergic reactions, decreased inflammation, bowel regulation, improved heart health and blood pressure, and decreased chance of colon cancer (Mercenier *et al.*, 2002). *Lactobacillus, Bifidobacterium,* and *Streptococcus* are three bacterial genus that are commonly used as probiotics; in addition, some *Enterococcus* species are also used (Argyri *et al.*, 2013). With increased consumer concern and awareness, the demand for natural products and alternatives to antibiotics have been increasing in today's society, thus warranting the abundance of research on

fermented food and lactic acid bacteria to control pathogen growth (Mercenier *et al.*, 2002).

Many researchers have successfully isolated lactic acid bacteria from fermented foods. *Lactobacillus plantarum* is one species of LAB that has strong probiotic properties and has been isolated from Korean fermented foods (Lim and Im, 2009). LAB with probiotic properties have also been isolated from fermented olives and identified as *Lactobacillus* species (Argyri *et al.*, 2013). It is important for LAB to be completely harmless to humans and it has been reported that LAB such as *Lactobacillus acidophilus* are capable of inhibiting the growth of pathogens while being harmless to the normal microbes in the human gastrointestinal tract (Fernandez *et al.*, 2003). It is also important for these gastrointestinal inhabitants to be able to survive the harsh conditions of the gut while possessing the ability to colonize. In Lim and Im's research study (2009), and in a study by Argyri *et al.*, (2013), *Lb. plantarum* was able to tolerate acid and bile, and showed strong adherence to intestinal cells. Other LAB species that have proven to have strong probiotic characteristics and have been commonly isolated are *Enterococcus faecium, Lactobacillus paracasei, Lactobacillus pentoses, Lactococcus lactis,* (Diop *et al.*, 2007; Argyri *et al.*, 2013).

One mechanism lactic acid bacteria use against pathogens is competitive exclusion. LAB compete with the pathogens for adherence to intestinal cells thereby

preventing the binding of some pathogens (Fernandez *et al.*, 2003). Another theory is that the presence of LAB can increase mucin production in the intestines which can either serve as a barrier or provide alternative receptors for pathogens (Ljungh and Wadstrom, 2006).

LAB in Broilers and *Salmonella*

By screening the bacterial community of animal intestines, many probiotics have been found (Heravi *et al*, 2011). "Broilers" are small young chickens, less than 8 weeks in age. The bacterial community in the ileum and cecum of broiler chicken contains different species of lactic acid bacteria. Interestingly, the relative percentages of those bacteria in the intestinal community change as the bird matures. Mostly, the highest percentage of *Lactobacillus* species bacteria, can be found in the ileum section of the intestine while the cecum is comprised mostly of *Clostridiaceae;* however, *Lactobacillus* can also be found in the cecum and *Clostridiaceae* in the ileum in smaller amounts (Lu *et al.*, 2003). As broilers age, the bacterial community present in its intestines does not only change but gets more and more complex; in addition, the bacterial community specifically in the ileum begins to vary more and more from that which is found in the cecum. These changes can depend largely on the diets of the broilers (Lu *et al*, 2003).

With respect to controlling bacterial contamination, the poultry production industry has relied on the use of antibiotics for many years; however, current research shows that antibiotic resistance can become a huge problem not only for broilers but for humans as well. LAB with antibiotic resistance can transfer that resistance to other bacteria in the gastrointestinal tract, making them difficult to kill in cases of foodborne illness. Therefore, with the increased chances of antibiotic resistance affecting humans, it is now important to find alternative ways to keep poultry safe for consumption. Although the use of antibiotics in broiler feed has been very common in the past, the use of probiotic LAB in feedings has been shown to have better effects on the chickens intestines and its normal micro flora. When compared with the effects of a probiotic feed, the feed containing antibiotics such as avilamycin, bacitracin and virginiamycin caused a significantly lower intestinal weight signifying that the antibiotics inhibited the growth of normal, beneficial microbes in the gut as well as pathogenic bacteria (Fajardo *et al.*, 2012, Patterson and Burkholder, 2003). LAB have been proven to contribute largely to broiler health and for building immunity while maintaining and protecting the normal microflora (Muir *et al.*, 2000).

A large amount of the foodborne illnesses are the direct result of contaminated poultry with *Salmonella* being one of the most commonly reported pathogens. Contamination of foods can come either directly or indirectly from poultry products (Doyle and Erickson, 2006) and some studies have shown that commercial poultry feed

can also be a source of contamination (Primm, 1998). Another source of contamination are flies and rodents (Adhikari *et al.*, 2004) as well as equipment used to transport the animals and also, manure (Doyle and Erickson, 2006). When broilers are being produced, pathogens normally found in fecal materials can accidentally come into contact with meat and other foods, thereby contaminating it. Consequently, if the proper food safety guidelines; such as Critical Control Points, are not followed throughout the entire production process, this contamination can ultimately cause illness in consumers (Stern *et al.*, 2006). Millions of Americans suffer from foodborne illness each year with over one million of those cases coming from *Salmonella* species alone (Mead *et al.*, 1999).

According to the Centers for Disease Control and Prevention (CDC), the *Salmonella* serotypes that most commonly cause infection are Enteritidis (18%), Typhimurium (13%), and Newport (13%) (2014). Outbreaks are commonly caused by egg products (CDC, 2000; Glynn *et al.*, 2004) and chicken consumption (Kimura et. al., 2004). Data from the CDC, Foodborne Diseases Active Surveillance Network (FoodNet) shows that the incidence of laboratory-confirmed *Salmonella* infections is the same as it was during the years 1996-1998, while the incidence of infections from *Listeria, Campylobacter, Yersinia*, and Shiga toxin-producing *Escherichia coli* have all decreased (2012).

Kimchi

"Kimchi" is a Korean traditional food that is fermented as a result of LAB activity (Park *et al.*, 2010) with the earliest documented use dating back to the 17th century. It has a sour and often spicy taste and is usually consumed cold as a side dish or as a healthy addition to other dishes like soups (Lee, 1997). There are many varieties of Kimchi and they are all made of many different vegetables including Asian cabbage (which has been salted and soaked) and radish. In addition to vegetables, Kimchi usually contains red pepper powder, garlic, ginger, fruit and some sort of fish product (optional) which are combined and placed between the cabbage leaves (Lee, 1997; Jeong *et al.*, 2013). Traditionally, the ingredients are stored for fermentation for a time period between 1 and 6 months where the pH drops to about 4.0 due to the production of organic acids.

The specifics of the fermentation process depend on the bacteria present from the ingredients. There are normally small amounts of LAB present in the raw ingredients of Kimchi and these LAB act as a starter culture for fermentation; as a result, lactic acid bacteria will eventually "dominate" the fermentation process (Kim *et al.*, 2002; Jung *et al.*, 2014). This process is also affected by fermentation temperature and concentrations of salt (Shin *et al.*, 1996; Lee *et al.*, 1992). There are different species of LAB that predominate at different points in the fermentation process. *Leuconostoc mesenteroides* is mostly present during the beginning of fermentation and is responsible for the decreased

pH during that time period; the next stage of the fermentation process shows a predominance of *Lactobacillus plantarum.* In addition to these two primary species, *Pediococcus* spp., *Weisella,*spp., and *Lactococcus* spp., are also important in the fermentation process (Lee *et al.*, 1992; Cheigh *et al.*, 1994; Park *et al.*, 2010). More specifically, *Leuconostoc citreum, Leu. carnosum, Leu. gasicomitatum, Leu. inhae, Leu.gelidum, Leu. kimchii, Leu. miyukkimchii, Lactobacillus sakei, Lb. brevis, Lb. curvatus, Weissella koreensis, W. cibaria, W. kimchii, W. soli* and *W. confuse* have been studied and characterized (Kim *et al.*, 2000; Lee *et al.*, 2002; Cho *et al.*, 2006). In early research studies, *Enterococcus, Pediococcus, and Streptococcus* spp. were thought to be the most dominant species in Kimchi fermentation however, now that identification methods have improved by 16S rDNA sequencing, those bacterial species have been less commonly identified and reported as having a lesser role in the fermentation process (Jung *et al.*, 2014).

Kimchi is increasing in popularity which could be due to the increased awareness of its many health benefits related to LAB and probiotics. Kimchi is known to not only contain large amounts of probiotics, but to also contain many other beneficial compounds such as antioxidants, vitamins, minerals and fiber (Park and Rhee, 2005). Some of the health benefits that have been reported include prevention and treatment of allergy symptoms, positive effects on diabetes issues, weight loss/ obesity treatment, decreased cholesterol, bowel and immune support (Lee, 1997; Islam and Choi, 2009; Han *et al.*,

2012; Ji *et al.*, 2012; Park *et al.*, 2012; Jang *et al.*, 2013). The antimicrobial properties of Kimchi due to LAB activity are a common area of study. Kimchi and the LAB it contains inhibit *Clostridium perfringens, Staphylococcus aureus, Listeria monocytogenes, Virbio parahaemolyticus, Escherichia coli* and *Salmonella* Typhimurium while allowing for LAB strains to increase and thrive (Ha, 1994).

Research Justification

The incidence of foodborne illness in the Unites States related to *Salmonella* spp. is not improving. According to the Centers for Disease Control and Prevention (CDC), many of the reported infections are a direct result of contaminated poultry and egg products. The health and lives of approximately 1.2 million people are affected by *Salmonella* each year. Although the use of antibiotics is popular, consumers are demanding more natural methods to treat and prevent these infections.

Lactic acid bacteria (LAB) are commonly used as probiotics and can be isolated from fermeneted food products as well as from the intestinal contents of broiler chickens. With American Kimchi, it is possible to find strains of LAB that have not been previously studied and therefore might show vital characteristics to increase their effectiveness as antimicrobial agents. In this study, potential probiotic LAB can be isolated and studied further. Ultimately, LAB that show strong probiotic characteristics can be applied to poultry feed to decrease the colonization of *Salmonella* spp. The bacteriocins that LAB produce, in addition to competitive exclusion, and additional compounds such as lactic acid, will make them ideal candidates for use as antimicrobial agents in poultry.

CHAPTER ONE

ISOLATION OF LACTIC ACID BACTERIA

Introduction

Lactic acid bacteria are generally recognized as safe (GRAS) for use in food products. They are oftentimes used as probiotics, which are defined as live microorganisms that offer a benefit to its host; usually by enhancing the micro flora of its intestines (Fuller, 1989). LAB that are commonly used as probiotics can be found in yogurt, cheese, sauerkraut, raw poultry meat, mulberries and many other fermented foods like *Plasom,* Sikhae and Kimchi (Lee *et al.*, 1997; Succi *et al.*, 2005; Hosseini *et al.*, 2009; Chen *et al*, 2010; Hwanhlem *et al.*, 2011).

Kimchi is made from a variety of vegetables which are then placed in refrigerated stored for fermentation, which is initiated by bacteria present in the raw ingredients. However, over time, lactic acid bacteria will "dominate" the fermentation process (Kim, 2002). *Leuconostoc* and *Lactobacillus* spp. are predominant genera in kimchi and can be successfully isolated. Although LAB are commonly found in fermented foods they are also known to colonize the intestines of broiler chicken, which are processed for human

consumption. Up to 70% of the bacteria in the ileum of a broiler chicken belong to the *Lactobacillus* genus, which is common among lactic acid bacteria (Lu *et al.*, 2003) Although antibiotics are currently used in animals and humans, to destroy pathogenic bacteria and prevent or treat illnesses, there is an increased demand from health-conscious consumers for more natural alternatives. The objective of this chapter is to isolate lactic acid bacteria from broilers and kimchi. My hypothesis is that LAB will dominate the fermentation process in our homemade American Kimchi and also that large amount of LAB colonize the intestines of the broilers that will be sampled in this study.

Materials and Methods

Kimchi Preparation

Three different types of Kimchi were made; red cabbage kimchi, white cabbage kimchi, and cucumber kimchi. First, the leaves of several Asian cabbages were dipped in water then spread with liberal amounts of salt and "soaked" for 4 hours. After soaking, the cabbage was then thoroughly rinsed; these salted cabbages were used as the base for the red cabbage kimchi and the white cabbage kimchi while salted, roughly chopped cucumbers were used as the base for the cucumber kimchi. Then, rice flour was mixed with water and heated until boiling (about 10 minutes). Chives, garlic, ginger, onion, pear, radish, and salt was added to the rice flour mixture then mixed well. This mixture

was then separated into three portions (one for each type of Kimchi). Red pepper powder and clam juice were added to the portions designated for the red cabbage kimchi and the cucumber kimchi, while mixture designated for the white cabbage Kimchi did not get these two ingredients. The finals mixtures were spread generously in between each of the cabbage leaves and throughout the cucumber pieces. Each Kimchi was placed in an individual sealed container and stored at 4°C for the fermentation process to begin. Homemade Kimchi's were prepared and stored in the Foods Lab at Delaware State University. Commercial kimchi was purchased from a local market in Newark, DE and stored at 4°C for sampling.

Isolation of Candidate LAB from Broilers and Kimchi Samples

Broiler chickens were obtained from the Agriculture department at the University of Maryland Eastern Shore. All broilers were euthanized with carbon dioxide gas (CO_2). Samples were taken from the ileum, cecum, and fecal materials of broilers at 7, 28, and 49 days old. 10-fold dilutions of each sample in MRS broth (Difco) with 0.05% L-Cysteine Hydrochloride (MRS+, Fisher Bioreagents, Fair Lawn, New Jersey, USA) were homogenized for 10 minutes using a stomacher machine (Interscience, St. Nom, France). 1ml aliquots were serial diluted with MRS broth, plated onto MRS+ agar (Difco) and cultured anaerobically for 48h at 37°C. Candidate LAB were isolated from each of the four types of Kimchi. Samples from homemade kimchi and commercial kimchi were

taken at 0, 10, 20, and 30 days of refrigerated storage. 25g of each Kimchi sample was homogenized with 225g MRS+ broth using a stomacher machine. 1ml aliquots of the homogenized mixtures were serial diluted with MRS broth, plated onto MRS+ agar and cultured anaerobically for 48h at 37°C. Isolated colonies from broiler and Kimchi samples that showed morphological properties that were characteristic of LAB, were selected and cultured individually in MRS+ broth for 24h. Each isolate was stored in 25% glycerol (Fisher Science) at -80°C as candidates in this study.

Results and Discussion

Isolation of Lactic Acid Bacteria

A total of 388 candidate LAB were isolated from Kimchi and Broiler samples combined; 194 from the Kimchi samples and 194 from the broiler samples. 62 isolates from cucumber kimchi, 60 from red cabbage kimchi, 34 from white cabbage kimchi, and 38 from the commercial kimchi. 77 candidates were isolated from the ileum of broiler chicken intestines, 83 from the cecum, and 34 from the fecal material. This result is shown in Table 1.

It was expected that the broiler chicken and kimchi would contain a variety of lactic acid bacteria as many previous studies have focused on species' of LAB from both sampling sources. Each isolate that was observed on the MRS agar had the morphology

that is characteristic of lactic acid bacteria isolated in previous studies. Figure 1 shows each of the prepared homemade Kimchi's, Cucumber, White Cabbage, and Red Cabbage, from left to right. Figure 2 shows cucumber kimchi in detail. Figure 3 shows three pictures taken during sampling at UMES chicken facility. Figure 4 shows the bacteria isolated from our initial 0 day fermentation sampling of the cucumber kimchi.

Table 1. Number of candidate LAB isolated from Kimchi and broiler samples.

Samples		Number of Isolates	
Broilers	Ileum	77	194
	Cecum	83	
	Fecal	34	
Kimchi	Cucumber	62	194
	Red	60	
	White	34	
	Commercial	38	

Figure 1. Homemade Kimchi's at 0 h fermentation. From left to right; Cucumber Kimchi made from salted cucumbers, White Cabbage Kimchi, and Red Cabbage Kimchi made from salted Asian cabbage. Kimchi's contain a mixture of vegetable ingredients.

Figure 2. Cucumber kimchi at 0 h fermentation. Ingredients mixed together include salted cucumber, red pepper powder, garlic, chives, onion, and clam juice.

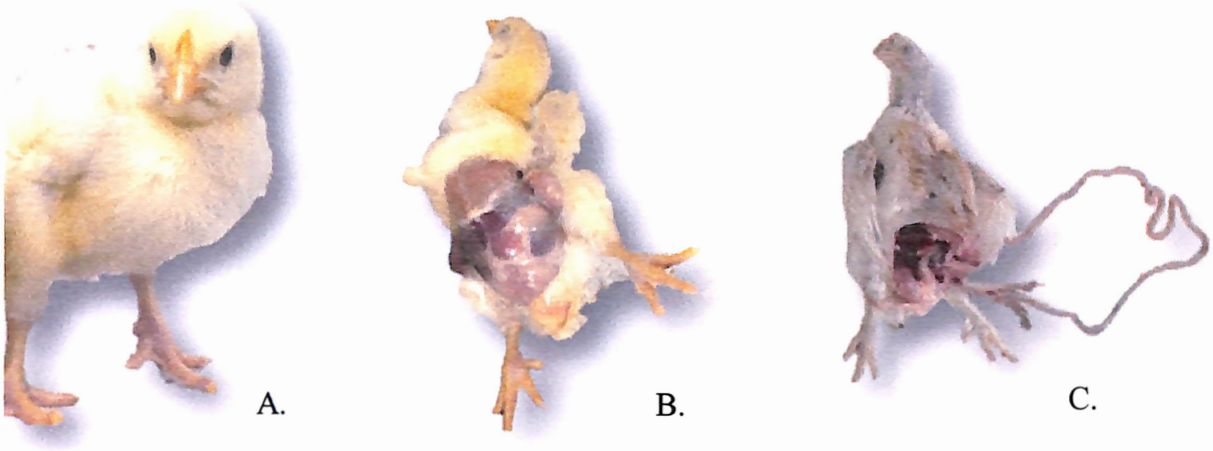


Figure 3. Broiler chickens sampled at The University of Maryland Eastern Shore. A: Broiler at 7d old. B: Internal organs of 7d old broiler. C: Ileum and cecum of 28d old broiler.

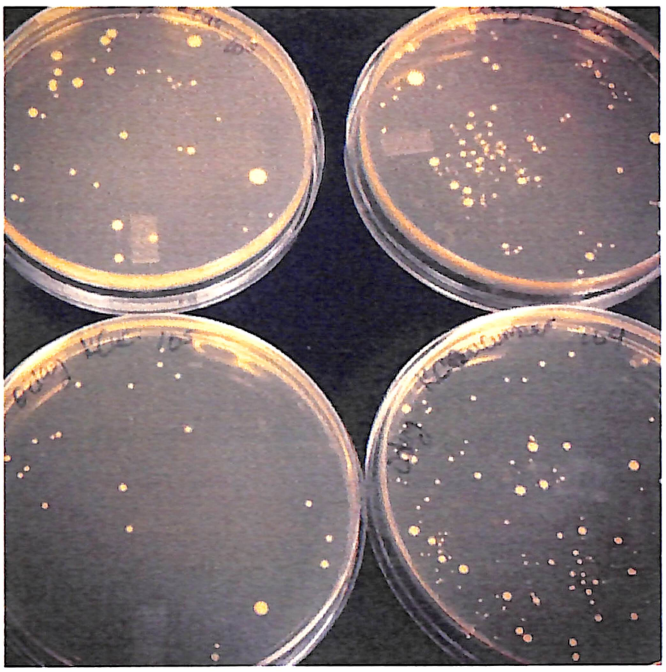


Figure 4. MRS agar plates with colonies isolated from cucumber kimchi. Figure shows two dilutions of initial bacterial colonies found in our cucumber kimchi at 0 days fermentation, in duplicate.

CHAPTER TWO

SCREENING FOR BACTERIOCIN PRODUCTION

Introduction

LAB are able to inhibit the growth of pathogens in a variety of ways. As the name suggests, LAB produce lactic acid, decreasing the pH of their environment to approximately pH 4, which is too low for some pathogens to survive. They also produce "bacteriocins", peptides synthesized in the ribosome of both gram-negative and gram-positive bacteria, which have the ability to inhibit the growth of a determined range of other bacteria. The killing range of bacteriocins produced by gram-positive bacteria is broader than that of other bacteriocins (Nes *et al.,* 2007) but more narrow that the inhibitory range of antibiotics.

Salmonella infections are responsible for millions of illnesses each year in the United States, with the majority of thses infections originating from poulty and egg products. Lactic acid bacteria that are isolated from Kimchi are known to produce bacteriocins that have antimicrobial activity against various *Salmonella* serotypes. Therefore, by screening lactic acid bacteria isolated, those possessing the strongest antimicrobial activity can be found and used in preventing infection. I hypothesize that the isolates in this study will produce bacteriocins that show antimicrobial activity against

Salmonella serotypes.. The objective of this chapter is to screen the isolates for lactic acid bacteria which can inhibit the growth of *S.* typhimurium, *S.* Heidelberg, *S.* Newport, and *S* enteritidis, by bacteriocin production.

Materials and Methods

Screening for LAB with Antimicrobial Activity Against *Salmonella* spp.

A modified assay of the well diffusion method used by Schillinger and Lucke (1989) was used to test each isolate for its ability to inhibit the growth of four Salmonella spp; *Salmonella* enterica serovar Typhimurium, Heidelberg, Enteritidis, and Newport; each obtained from The United States Department of Agriculture. Each strain was activated using Tryptic Soy Agar (TSA, Carolina Biological Supply Co., Burlington NC); Strains were plated and incubated for 24h at 37°C. Cultures of each strain were then grown in tryptic soy broth (TSB, Carolina Biological Supply Co., Burlington NC) for 24h then adjusted to 0.3OD at 600nm using a micro plate reader (Biotech Instruments Inc., Winooski, VT). 0.5% of each strain was inoculated into 0.7% nutrient agar (Carolina Biological Supply co.) that had cooled to about 50°C then after gently swirling, 5ml of top agar was poured onto prepared nutrient agar plates. After setting for about 15 minutes, 3mm wells were made into the agar then 15 µl of each isolate was placed into the wells. The plates were incubated anaerobically at 37°C for 48h. After incubation, the diameter of any clear zones present were measured to the nearest mm.

Screening for LAB Producing Bacteriocins Active Against *Salmonella* spp.

The LAB isolates that showed clear zones for *Salmonella* spp. were tested further for production of bacteriocin by the top agar/well diffusion method. Each isolate was grown in MRS broth + L-cysteine for 24h. The cell-free supernatant was obtained by centrifuging the cells at 3000×g for 10minutes. The supernatant was adjusted to pH 6.5 with the addition of 2M sodium hydroxide (Fisher Science) to eliminate the effect of any organic acids produced. Then 1 mg/ml catalase was added to eliminate the effect of any hydrogen peroxide that is produced by the strains. Then the cell-free supernatant was filter sterilized using 0.22μm pore size syringe filter (Millipore, Billerica, MA.) and used as crude bacteriocin. On the *Salmonella*-inoculated nutrient agar mentioned previously, 6mm wells were made and 100μl of crude bacteriocin was added to each well. After incubation for 12 h at 37°C, the diameter of any clear zones were measured. LAB that produced bacteriocin inhibiting the growth of *Salmonella* spp. were selected for probiotic characterization.

Results and Discussion

Antimicrobial Effect of Isolated LAB Against *Salmonella* spp.

A total of 132 isolated LAB strains showed antimicrobial effect against 1 or more of the following *Salmonella* strains; *S.* Typhimurium, *S.* Heidelberg, *S.* Enteritidis, *S.*

Newport. LAB isolates that showed a clear zone greater than 6mm in diameter were considered positive for *Salmonella* inhibition. An example of the clear zones produced is shown in Figure 5. The isolates from red cabbage kimchi and from the ileum of the broilers, showed the highest number of potential probiotic strains. Among the four tested *Salmonella* strains, there was an average of 34.25 isolates from the ileum that exhibited antimicrobial effects, and an average of 22.5 from red cabbage kimchi; whereas, the average number of positive LAB among the other broiler and Kimchi isolates ranged from 8.25 to 17.25.

The supernatant from the isolates was then obtained for screening of bacteriocin production. Among the LAB positive for *Salmonella* inhibition, 50 showed a cell free antibacterial effect against 1 or more of the *Salmonella* strains; A total of 23 isolates from broiler intestines (8 isolated from the ileum, 4 from the cecum, 11 from the fecal material) and 27 from the Kimchi's (4 from cucumber kimchi, 7 from red cabbage, 3 from white cabbage, and 13 from commercial kimchi. These results can be found in table 2. Two of the Kimchi isolates, I3-3 and Cuc-77 produced compounds that showed a significant zone of inhibition for *Salmonella* spp, larger than that of any other isolates. Supernatant from I3-3 showed zones of inhibition greater than 13mm for all four *Salmonella* spp. and the supernatant from Cuc-77 showed similar results for three of the four tested *Salmonella* strains (its activity toward *S.* Newport was slightly less strong at 10-12mm in diameter. There were a total of 10 isolates (6 from broilers and 4 from Kimchi) which showed bacteriocin-like effects against all four of the tested *Salmonella*

strains (C3-13, C3-15, Cab-37, Cuc-66, Cuc-77, I1-57, I2-31, I3-3, F-50, and W-51). Some isolates showed a very weak result, only showing inhibition of one *Salmonella* strain and/or showing a very small zone of inhibiton < 9mm. The detailed results of the crude bacteriocin *Salmonella* inhibition can be found in Table 3.

It has been shown that gram positive bacteria such as *Lactobacillus acidophilus* have the ability to inhibit gram negative bacteria (Tagg, Dajani, and Wannamaker, 1976) which agrees with the results from this experiment. Interestingly, it has been reported that after neutralization of the LAB's cell-free supernatants to pH 6.5, all inhibitory effects were lost (Argyri *et al.*, 2013; Lin *et al.*, 2007; Maragkoudakis et. al., 2006) which contrasts the results obtained here. From this experiment, potential probiotic LAB were successfully screened from those LAB that did not possess a strong anti-*Salmonella* ability. In addition, the LAB that we have isolated, showed inhibitory effect against the most commonly reported species of *Salmonella* in foodborne illness and outbreaks (CDC, 2014) by bacteria and bacteriocin. Bacteriocins are known to be active against a more narrow range of bacteria than that of traditional antibiotics (Zacharof and Lovitt, 2012) showing a lesser chance of complete wipeout of the intestinal microflora. All positive isolates were further tested for their probiotic characteristics

Table 2. Total LAB isolated and number of isolates with antimicrobial effects against *Salmonella*.

	Sample	Number of isolates	Number of antimicrobial isolates	Number of bacteriocin- producing isolates
Kimchi	Red cabbage	60	26	7
	White cabbage	34	9	3
	Cucumber	62	15	4
	Commercial	38	22	13
Broiler chicken	Ileum	77	36	8
	Cecum	83	9	4
	Fecal	34	15	11
Total		388	122	50

Table 3. Antibacterial activities of crude bacteriocin produced from Kimchi isolates and broiler isolates against 4 *Salmonella* strains.

Isolate	*S.* Enterit.	*S.* Heidelberg	*S.* Newport	*S.* Typhim.	Isolate	*S.* Enterit	*S.* Heidelberg	*S.* Newport	*S.* Typhim.
Cab-18	+	+	-	+++	Com 75	++	-	-	+
Cab-21	+++	+	-	+	Com 77	-	+	-	-
Cab-25	++	+++	-	-	I1-57	++	++	++	++
Cab-37	++	+	+	+++	I2-1	++	-	+	-
Cab-39	-	-	-	+++	I2-15	-	-	-	+
Cab-50	-	-	+	+	I2-31	+++	++	+	++
Cab-78	+	-	-	-	I3-2	++	-	-	++
W-51	+	+	+	++	I3-3	+++	+++	+++	+++
W-53	-	-	-	+	I3-7	++	-	-	-
W-71	-	+	-	++	I3-32	+	-	-	-
Cuc-1	+	-	++	++	C2-30	-	++	-	+++
Cuc-52	+++	++	-	+	C3-12	+++	-	-	-
Cuc-66	+++	++	++	+	C3-13	+++	++	+	+
Cuc-77	+++	+++	++	+++	C3-15	+++	++	+	+++
Com-3	+	-	-	-	F-1	++	++	-	++
Com-33	+	-	-	-	F-6	++	++	-	-
Com-34	-	+	-	-	F-11	++	-	-	-
Com-35	+++	+++	-	-	F-34	++	++	-	++
Com-36	+	++	-	-	F-50	++	+	+	++
Com-53	-	+	-	-	F-54	+	+	+	-
Com-54	+++	+	+	-	F-56	-	+	-	-
Com-62	-	+	-	-	F-57	+	+	-	-
Com-72	-	-	-	+	F-58	-	-	+	-
Com-73	+	-	+	++	F-59	+	+	++	-
Com-74	+	+	+	-	F-60	+	-	-	-

+ shows positive result of clear zone 9mm or less. ++ shows positive result of clear zone 10-12mm.+++ shows positive result of clear zone 13mm or greater. – shows negative result.

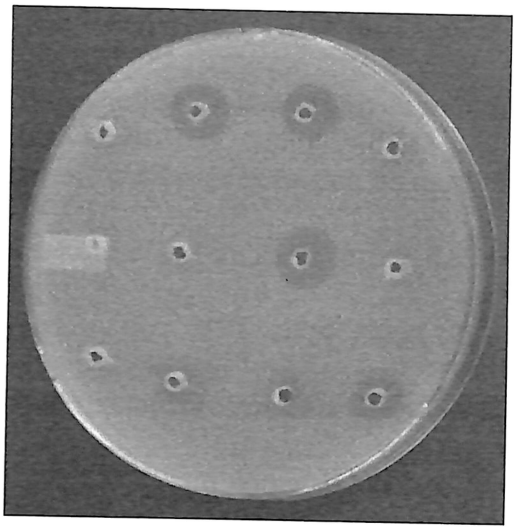


Figure 5. Inhibition results toward *S.* enteritidis by LAB isolates on nutrient agar.

CHAPTER THREE

PROBIOTIC CHARACTERIZATION AND BACTERIA IDENTIFICATION

Introduction

Lactic acid bacteria, whether isolated from fermented foods such as Kimchi or isolated from the intestines of broiler chicken, can be used as probiotics for humans and animals respectively. Therefore, it is important to test these bacteria to ensure that they have probiotic characteristics that will allow them to survive and thrive in the gastrointestinal tract of their host. Researchers have tested potential probiotic LAB against bile salt, acidic conditions, antibiotics as well as testing their adherence ability (Argyri *et al.*, 2013, Lim and Im, 2009) but no research has been done on LAB isolated from American-made kimchi.

Antibiotics are widely used in poultry production, usually, for one or more of four main reasons; for treatment, control, prevention, or growth. When antibiotics are not administered correctly, pathogens can become resistant creating a bigger problem (AMI, 2014), the threat of antibiotic resistance. Lactobacillus lactis for example is known to carry antibiotic resistance genes and can transfer resistance to harmful bacteria by horizontal gene transfer (HGT) (Wang et. al., 2006). Therefore it is important to determine the antibiotic susceptibility of LAB that are indended as probiotics.

My hypothesis is that some of the isolated bacteria will have strong probiotic characteristics and some will not. Also, I believe that some bacteria will posses antibiotic resistance and will therefore need to be eliminated further consideration as probiotic candidates. The objective of this chapter is to determine the probiotic abilities of the isolated LAB and to identify the isolates with the strongest probiotic potential

Materials and Methods

Bile Salt and Gastric Juice Tolerance

Each isolate capable of producing bacteriocin active against *Salmonella* spp. was tested for its tolerance of bile salt and gastric juice. The method used by Lim and Im (2009) was modified and used for the gastric juice and bile salt assays. For the bile salt tolerance assay, 0.3% bile salt (Fisher Science Education, Nazareth, PA) was added to MRS broth. For the gastric juice assay, phosphate buffered saline (PBS) was adjusted to pH 2.5 using hydrochloric acid then pepsin (Acros Organics, New Jersey) was added at 3mg/ml. 1% of each isolate (0.4 OD at 600nm) was inoculated into the gastric juice and bile solutions then incubated at 37°C. Colony forming units (CFU) were enumerated at 0h and 2h for the gastric juice assay and 0h and 24h for the bile salt assay.

Enzyme Resistance

All bacteriocin-producing isolates were tested for their ability to resist the effect of enzymes. Enzyme-MRS broth solutions were prepared; one for each of the following enzymes; 1mg/ml of alpha amylase (MP Biomedicals, Solon, OH) and 0.5mg/ml lysozyme (Fisher Bioreagents, Fair Lawn, New Jersey). 48h growth cultures of each LAB were inoculated into each enzyme-MRS solution at 0.5% and also into a control MRS without the addition of any enzymes. Optical densities (600nm) were measured using a micro plate reader at 0 h, 4 h, and 24 h of incubation at 37°C. Isolates showing enzyme tolerance by OD readings, were then enumerated by plate count method. Enzyme tolerance of the LAB isolates for both enzymes were compared with the control.

Antibiotic Susceptibility

The susceptibility of each isolate to six different antibiotics was tested using the agar/well diffusion method. 0.7% MRS top agar that had been inoculated with 0.3% of each LAB strain was poured onto MRS agar plates. 3mm wells were made into the agar then 10µl of erythromycin (1mg/ml MP Biomedicals, LLC, Solon, OH), ampicillin (3mg/ml Alfa Aesar, Heysham, Lancs.), streptomycin (5mg/ml MP Biomedicals, Solon, OH), kanamycin (5mg/ml Boston Bioproducts), chloramphenicol (3mg/ml Alfa Aesar, Ward Hill, MA), and tetracycline (3mg/ml MP Biomedicals, LLC, Solon, OH) were

placed into individual wells. Plates were incubated anaerobically for 24h at 37°C then the diameter of any clear zones were measured.

Sequencing Analysis of Probiotic LAB Isolates

LAB isolates were selected by their bacteriocin production and probiotic characteristics. These isolates were identified by 16S rDNA sequencing performed at Genewiz, Inc. (South Plainfield, NJ). The sequences obtained were analyzed with the BLAST program of the National Center for Biotechnology Information (NCBI; Bethesda, MD). The BioEdit v. 7.2 program (Tom Hall Ibis Therapeutics, Carlsbad, CA) was used to optimize sequences and CLUSTAL W (http://www.genome.jp/tools/clustalw/) was used for alignment of the sequences. The neighbor-joining method was used for constructing a phylogenetic tree and conducted with the MEGA v. 6 (Tamura *et al.* 2013). Confidence values for individual nodes were determined by 1,000 replication bootstrap analyses.

Results and Discussion

Bile Salt and Gastric Juice

Two of the most harsh conditions of the body, that could potentially decrease the effectiveness of probiotics is the gastric environment created in the stomach and the bile

salt excreted by the liver. The low pH of the stomach and the effect of bile can prevent some bacteria from maintaining viability throughout the gastrointestinal tract, rendering them useless in the colon (Casey et. al., 2004). If administered orally, probiotics must travel through the stomach where the pH can get as low as about 2.0 therefore we tested the ability of the isolates to maintain viability after exposure to pH 2.0 for two hours. The survival of probiotics through these conditions allow for them to successfully colonize and thrive. Among the 50 tested candidate LAB strains, 48 were able to survive in the presence of bile salts while 36 strains were able to survive in the presence of the gastric environment. After 2 hours of incubation with gastric juice, 8 isolates were able to survive at Log 5 CFU or higher; those isolates are I2-15, I3-3, I3-7, F-11, F-6, Com-72, and Com-3. After 24 hours of incubation with bile salt, F-54, Cab-18, and Cab-25 were able to continue to grow and reach a Log CFU higher than their initial count showing the greatest resistance. These results are in shown in detail in Tables 4a, 4b, 5a and 5b .

Similar results were obtained in the study by Casey et. al., where all of the isolates (from the feces and cecum) were able to tolerate bile in concentrations up to 0.3% and the survivability of the isolates in gastric juice was variable (2004). It is known that *Lactobacillus. acidophilis* is a strong survivor of gastric acid (Marteau *et al.*, 1997) and also that *Bifidobacterium bifidum* as well as *Lactobacillus acidophilus* were able to survive a gastric model whereas *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were not able to survive (Marteau, Minekus, Havenaar, and Huis In't Veld, 1997). The isolates in this study that were survivors of both gastric juice and bile were

considered to be the strongest candidates for probiotic use and those that were not able to survive in these experiments were eliminated and were not further considered as candidates for probiotic use.

Effect of Enzymes

Among the screened isolates, 16 candidates were able to grow well in the presence of enzymes alpha amylase and lysozyme. There were 9 LAB, isolated from Kimchi samples, which were the strongest among the isolates. Cab-18, the strongest isolate, isolated from red cabbage Kimchi, contained an average of 9.7 log CFU per ml after enzyme exposure. Com-26 was unable to survive the treatment of lysozyme. Enzymes in the mouth such as alpha-amylase, can destroy lactic acid bacteria administered orally therefore it is important that alpha-amylase resistance is a characteristic of any lactic acid bacteria to be used a probiotics. The positive results are shown in Figure 6.

Antibiotic Susceptibility

The well diffusion method produced clear zones of inhibition around those wells containing LAB that are susceptible to antibiotics. An example is shown in Figure 7. Among 50 tested isolates, the following 12 were susceptible to all six tested antibiotics,

W-53, W-71, Cuc-52, Cuc-66, Com 33, Com 34, Com 54, Com-72, Com-73, Com-74, Com-75, and F-54 which is demonstrated by the presence of all six clear zones. All isolates were susceptible to the antibiotic tetracycline except com-36 and com-62. All isolates were susceptible to the antibiotic erythromycin except I2-1 and I3-2. The results are show in Table 6. Isolated LAB that have resistance to antibiotics can be dangerous to humans.

There are varying opinions when it comes to the subject of antibiotic susceptibility. On one hand, it has been said that antibiotic resistance can be transferred from LAB to other bacteria that might enter the human body (Curragh and Collins, 1992). Horizontal gene transfer (HGT) has been previously seen in poultry relating to Salmonella serotypes and is known as a potential risk to humans (Johnson et al., 2010). Transfer of antibiotic resistance genes *tet*(M) and *erm*(B) responsible for tetracycline and erythromycin resistances in lactic acid bacteria has been studied (Nawaz et. al., 2011). If the resistance is transferred to a pathogenic bacterial strain, then it will become very difficult for the person to be treated for illnesses. On the other hand, some antibiotics might cause gastrointestinal upset and diarrhea. In this case having probiotics with antibiotic resistance present in the colon could potentially be beneficial (Charteris *et al.*, 1998). As shown, all LAB isolated in this study were susceptible to tetracycline except for 2 strains, while 12 of the LAB isolated in this study showed no antibiotic resistance at all. This means that they would not pose any threats in regards to transferring antibiotic resistance to other bacteria making them potentially safer for use in humans.

Sequencing, Analysis and Identification of Final Candidates

The LAB strains that displayed the strongest probiotic characteristics as shown by the previous experiments were identified by 16S rDNA sequencing. From Kimchi, Cab-18 and Cab 25 were identified as *Lactobacillus casei*. Cab 39 was identified as *Lactobacillus plantarum*. Com 36 was identified as *Lactobacillus sakei*. Com-54 was identified as *Leuconostoc mesenteroides*. Cuc-1 was identified as *Lactobacillus saniviri*. It had been previously proven that *Lactobacllus plantarum* is one of the most dominant bacteria in the final stages of kimchi fermentation; therefore, isolation of that bacterial strain suggests the homemade kimchi was successfully fermented. From broilers W-51 was identified as *Leuconostoc mesenteroides*. I2-31, C3-15, F-1, F-50, and F-59 were identified as *Lactobacillus crispatus*. F-6 was identified as *Lactobacillus johnsonii*. The Genbank accession numbers are KF263164.1, KF149523.1, and JN644756.1 respectively. These results are shown in Table 7. The phylogenetic tree of the identified isolates can be found in Figure 8.

Table 4.a. Tolerance of screened broiler isolates to artificial gastric juice at 0 h and 2 h of incubation. Values represent Log_{10} of CFUs/ml.

Isolate	0 h	2 h
I3-32	5.59 ± 0.16	-
I2-15	5.15 ± 0.21	5.06 ± 0.07
I1-57	5.84 ± 0.09	-
I2-31	6.01 ± 0.15	2.48 ± 0.45
I2-1	5.30 ± 0.00	-
I3-2	6.03 ± 0.11	-
I3-3	6.36 ± 0.11	5.91 ± 0.19
I3-7	5.93 ± 0.04	5.95 ± 0.00
C2-30	5.80 ± 0.28	2.88 ± 0.10
C3-13	5.81 ± 0.05	1.45 ± 0.21
C3 15	6.45 ± 0.04	-
C3-12	6.42 ± 0.14	2.46 ± 0.04
F-34	6.05 ± 0.38	3.15 ± 0.21
F-50	6.35 ± 0.07	2.74 ± 0.06
F-11	6.15 ± 0.04	6.27 ± 0.02
F-6	5.94 ± 0.34	6.20 ± 0.04
F-56	6.25 ± 0.24	4.13 ± 0.18
F-57	6.53 ± 0.19	3.60 ± 0.43
F-1	6.05 ± 0.14	2.71 ± 0.03
F-54	5.80 ± 0.14	3.66 ± 0.26
F-59	6.15 ± 0.11	4.03 ± 0.11
F-58	6.29 ± 0.12	6.57 ± 0.03
F-60	5.90 ± 0.00	2.74 ± 0.01

Table 4.b. Tolerance of screened Kimchi isolates to artificial gastric juice at 0 h and 2 h of incubation. Values represent Log_{10} of CFUs/ml.

Isolate	0 h	2 h
Cab-37	6.97 ± 0.01	-
Cab-25	6.43 ± 0	1.58 ± 0.15
Cab-50	6.30 ± 0	2.13 ± 0.13
Com-35	6.63 ± 6.63	-
Com-75	7.16 ± 0.01	3.45 ± 0.21
Com-73	6.92 ± 0.11	2.22 ± 0.37
Com-54	7.08 ± 0.00	4.02 ± 0.17
Com-62	6.03 ± 0.11	3.58 ± 0.15
Com-74	7.25 ± 0.02	4.25 ± 0
Com-72	6.93 ± 0.02	5.14 ± 0
Com-36	6.34 ± 0.19	3.92 ± 0.10
Com-34	6.86 ± 0.08	-
Com-53	6 ± 0.42	-
Com-77	7.13 ± 0.05	-
W-51	7.33 ± 0.05	1 ± 0
Cab-39	6.79 ± 0.03	3.45 ± 0.21
Com-33	7.06 ± 0.03	1.34 ± 0.49
Com-3	7.12 ± 0.06	5.22 ± 0.05
Cab-78	7.22 ± 0.00	-
W-71	7.19 ± 0.00	-
Cab-21	6.69 ± 0.07	1.77 ± 0.10
W-53	7.19 ± 0.04	1 ± 0
Cab-18	6.81 ± 0.00	2.47 ± 0.04
Cuc-66	6.51 ± 0.05	1.47 ± 0
Cuc-52	7.21 ± 0	-
Cuc-1	6.67 ± 0.07	3.84 ± 0
Cuc-77	6.32 ± 0	-

Table 5.a. Tolerance of screened broiler isolates to bile salt at 0 h and 24 h of incubation.

Values represent Log_{10} of CFUs/ml.

Isolate	0 h	24 h
I2-15	6.11 ± 0.29	5.45 ± 0.21
I1-57	6.47 ± 0	4.48 ± 0.17
I2-31	6.06 ± 0.15	4.66 ± 0.11
I2-1	6.21 ± 0.01	4.80 ± 0.01
I3-2	6.50 ± 0.17	5.75 ± 0.21
I3-3	6.09 ± 0.02	5.38 ± 0.12
I3-7	6.23 ± 0	4.33 ± 0.08
C2-30	6.16 ± 0.16	5.14 ± 0.03
C3-13	6.37 ± 0.07	6.17 ± 0
C3 15	6.48 ± 0.03	4.11 ± 0.22
C3-12	6.37 ± 0.07	5.81 ± 0.04
F-34	6.21 ± 0.05	5.03 ± 0.00
F-50	6.41 ± 0.04	-
F-11	6.46 ± 0.01	4.21± 0.18
F-6	6.05 ± 0.13	5.47± 0
F-56	6.26 ± 0.13	3.58 ± 0.15
F-57	6.12 ± 0.018	5.77± 0.24
F-1	6.53 ± 0.02	4.70 ± 0.00
F-54	6.67 ± 0.03	7.03 ± 0.01
F-59	6.41 ± 0.10	6.22 ± 0.07
F-58	6.40 ± 0.06	3.97± 0.03
F-60	6.57 ± 0.04	4.92 ± 0.02

Table 5.b. Tolerance of screened Kimchi isolates to bile salt at 0 h and 24 h of incubation. Values represent Log_{10} of CFUs/ml.

Isolate	0 h	24 h
Cab-37	6.88 ± 0.02	5.23 ± 0.00
Cab-21	6.64 ± 0.08	6.40 ± 0.01
Cab-18	6.84 ± 0.05	7.09 ± 0.00
Cab-25	6.97 ± 0.08	7.00 ± 0.33
Cab-50	6.78 ± 0.07	6.04 ± 0
Cab-39	6.78 ± 0.02	6.04 ± 0
Com-35	5.62 ± 0.21	-
Com-75	7.19 ± 0.01	6.02 ± 0.02
Com-73	7.13 ± 0.04	6.43 ± 0.03
Com-54	6.92 ± 0.01	5.80 ± 0.28
Com-62	5.77 ± 0.10	5.38 ± 0.12
Com-74	7.21 ± 0.02	6.35 ± 0.21
Com-72	6.98 ± 0.02	5.73 ± 0.05
Com-36	6.20 ± 0.13	4.85 ± 0.04
Com-34	7.06 ± 0.11	4.74 ± 0.06
Com-53	6.25 ± 0	4.36 ± 0.02
Com-77	7.15 ± 0.00	5.96 ± 0.01
Com-33	6.07 ± 0.05	3.45 ± 0.21
Com-3	7.17 ± 0.03	5.08 ± 0.05
Cab-78	7.27 ± 0.00	6.42 ± 0.03
W-51	7.18 ± 0.04	6.25 ± 0.03
W-53	7.14 ± 0.09	6.07 ± 0.05
W-71	7.18 ± 0.05	5.94 ± 0.13
Cuc-52	6.67 ± 0.01	5.77 ± 0.10
Cuc-66	6.21 ± 0.05	5.92 ± 0.16
Cuc-77	5.95 ± 0.06	5.62 ± 0.21

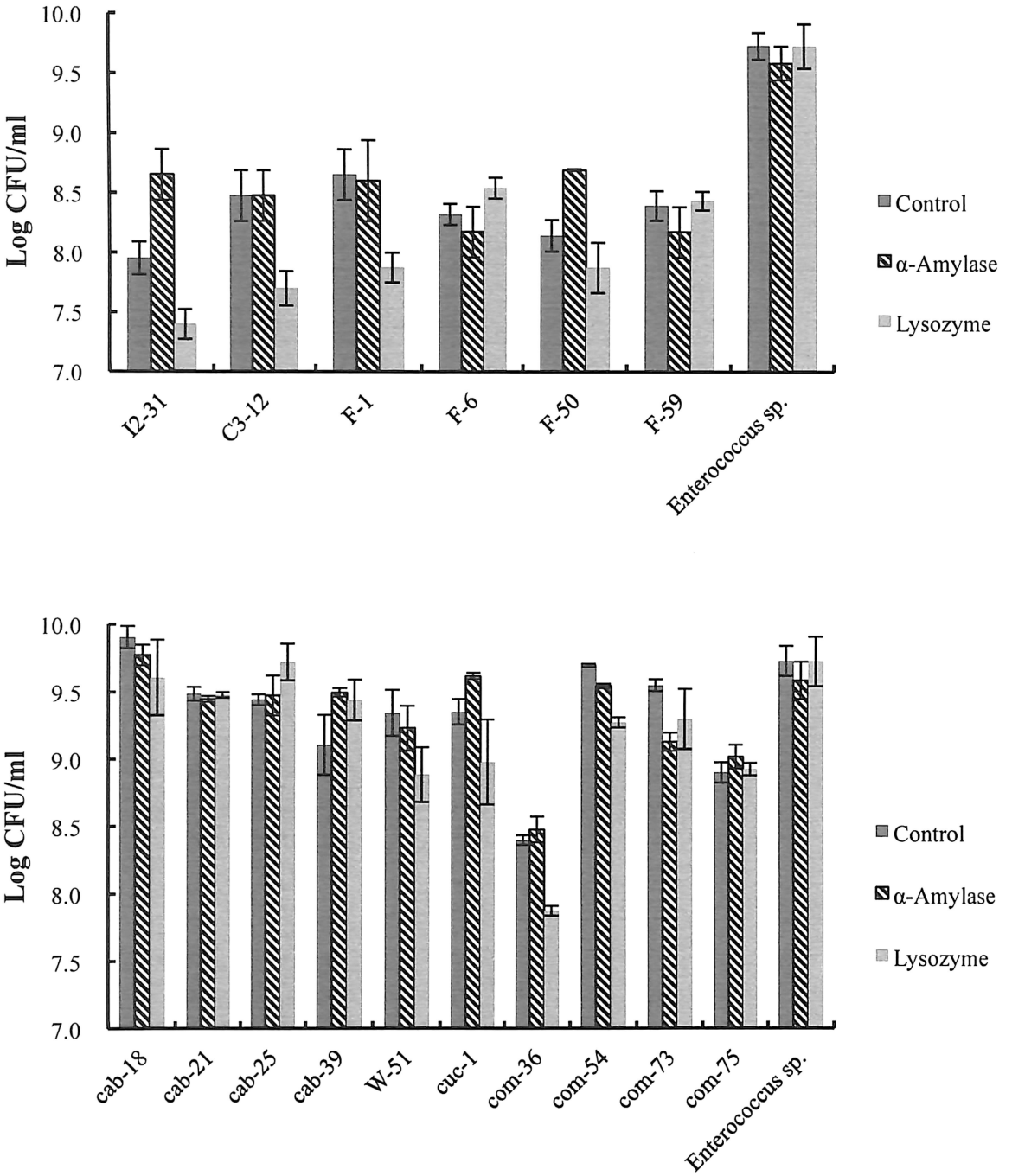


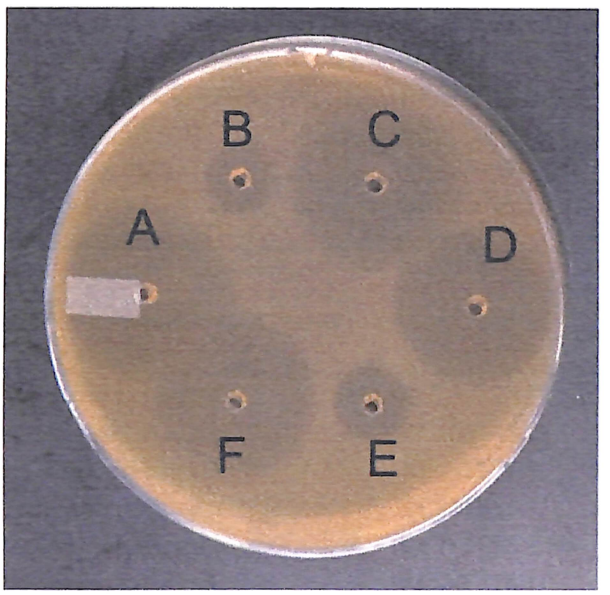
Figure 6. Enzyme effect on bacteria isolate from broilers (A) and Kimchi (B). All tested LAB isolates were able to tolerate enzymes as compared with control

Table 6. Antibiotic susceptibility of isolated LAB

Isolates	Ampicilin	Chloramphenicol	Erythromycin	Kanamycin	Streptomycin	Tetracycline
Cab-18	+	+++	+++	-	-	+++
Cab-21	+++	+++	+++	-	-	++
Cab-25	++	+++	+++	-	-	++++
Cab-37	++	+++	++	-	+	++
Cab-39	++	++	++	-	-	++
Cab-50	++	++	++	-	-	+++
Cab-78	++++	+++	+++	-	-	++
W-51	++	+++	+++	-	-	+++
W-53	++	+++	+++	+	+	+++
W-71	+++	+++	+++	+	++	+++
Cuc-1	++	++	++	-	-	+++
Cuc-52	++	+++	+++	+	+	+++
Cuc-66	+	+++	+++	+++	++	+++
Cuc-77	+	++	++	-	-	+++
Com-3	++	+++	+++	-	+	+++
Com-33	++	++	++	+	++	+++
Com-34	++	+++	+++	+	+	+++
Com-35	+	++++	+++	-	-	++++
Com-36	+	+++	+++	-	-	-
Com-53	+	+++	+++	-	-	+
Com-54	++	+++	+++	+	+	+++
Com-62	+	+++	+++	-	-	-
Com-72	++	+++	+++	+	++	+++
Com-73	++	+++	+++	+	+	+++
Com-74	++	+++	+++	+	+	+++
Com-75	++	+++	+++	+	+	+++
Com-77	++	+++	+++	-	+++	++
I1-57	++	+++	+++	-	++	+++
I2-1	+++	+++	-	-	++	++++
I2-15	++	+++	+++	-	++	+++
I2-31	++	+++	+	-	++	+++
I3-2	++	+++	-	-	++	+++
I3-3	++	+++	+++	-	++	++++
I3-7	+++	++++	+++	-	++	++++
I3-32	++	+++	+++	-	+	++++
C2-30	+++	+++	+	-	-	+++
C3-12	++	+++	+++	-	++	+++
C3-13	+++	+++	+++	-	++	+++
C3-15	+++	+++	+++	-	+	++++
F-1	+++	+++	+++	-	++	++++
F-6	+++	+++	++++	-	++	++++
F-11	++	+++	+++	-	-	+++
F-34	+++	+++	+++	-	++	+++
F-50	+++	+++	+++	-	-	+++
F-54	+	+++	+++	+	+	+++
F-56	++	++++	+++	-	-	++
F-57	++	++	++	-	+	+++
F-58	+++	+++	+++	-	-	+++
F-59	++	+++	+++	-	++	+++
F-60	+++	++++	++	-	-	++
Enterococcus sp.	+	+++	+++	-	-	++

+ represents ≤ 10mm ++ represents 11-20mm +++ represents 21-30mm ++++ >30mm – represents absence of inhibition.

Figure 7. Antibiotic susceptibility of white kimchi isolate W-71



A: Ampicillin, B: Kanamycin, C: Chloramphenicol D: Erythromycin, E: Streptomycin, F: Tetracycline. W-71 results showed susceptibility to all 6 tested antibiotics.

Table 7. Identification of bacteriocin producing probiotic LAB strains isolated from Kimchi and broiler chicken

Origin	Isolates	Identified species	Accession #	Identity	Blasted length (bp)
Kimchi	Cab-18	*Lactobacillus casei*	KF263164.1	100	573
		Lactobacillus paracasei	KC967212.1	100	
	Cab-21	*Leuconostoc pseudomesenteroides*	KF263165.1	99	793
	Cab-25	*Lactobacillus casei*	KF263160.1	99	726
		Lactobacillus paracasei	KF263163.1	99	
	Cab-39	*Lactobacillus plantarum*	KF767997.1	100	792
	W-51	*Leuconostoc mesenteroides*	KF149523.1	99	566
	Cuc-1	*Lactobacillus saniviri*	AB602569.1	99	577
	Com-36	*Lactobacillus sakei*	HG798441.1	99	694
	Com-54	*Leuconostoc mesenteroides*	KF149523.1	98	593
	Com-73	*Leuconostoc mesenteroides subsp. mesenteroides*	HG799977.1	100	755
	Com-75	*Leuconostoc mesenteroides subsp. mesenteroides*	HG799977.1	99	833
Broiler chiken	I2-31	*Lactobacillus crispatus*	KC747723.1	99	849
	C3-12	*Lactobacillus crispatus*	KC166145.1	100	781
	F-1	*Lactobacillus crispatus*	KF661284.1	100	847
	F-6	*Lactobacillus johnsonii*	KC856466.1	100	829
	F-50	*Lactobacillus crispatus*	KC747723.1	99	850
	F-59	*Lactobacillus crispatus*	KC747723.1	99	843

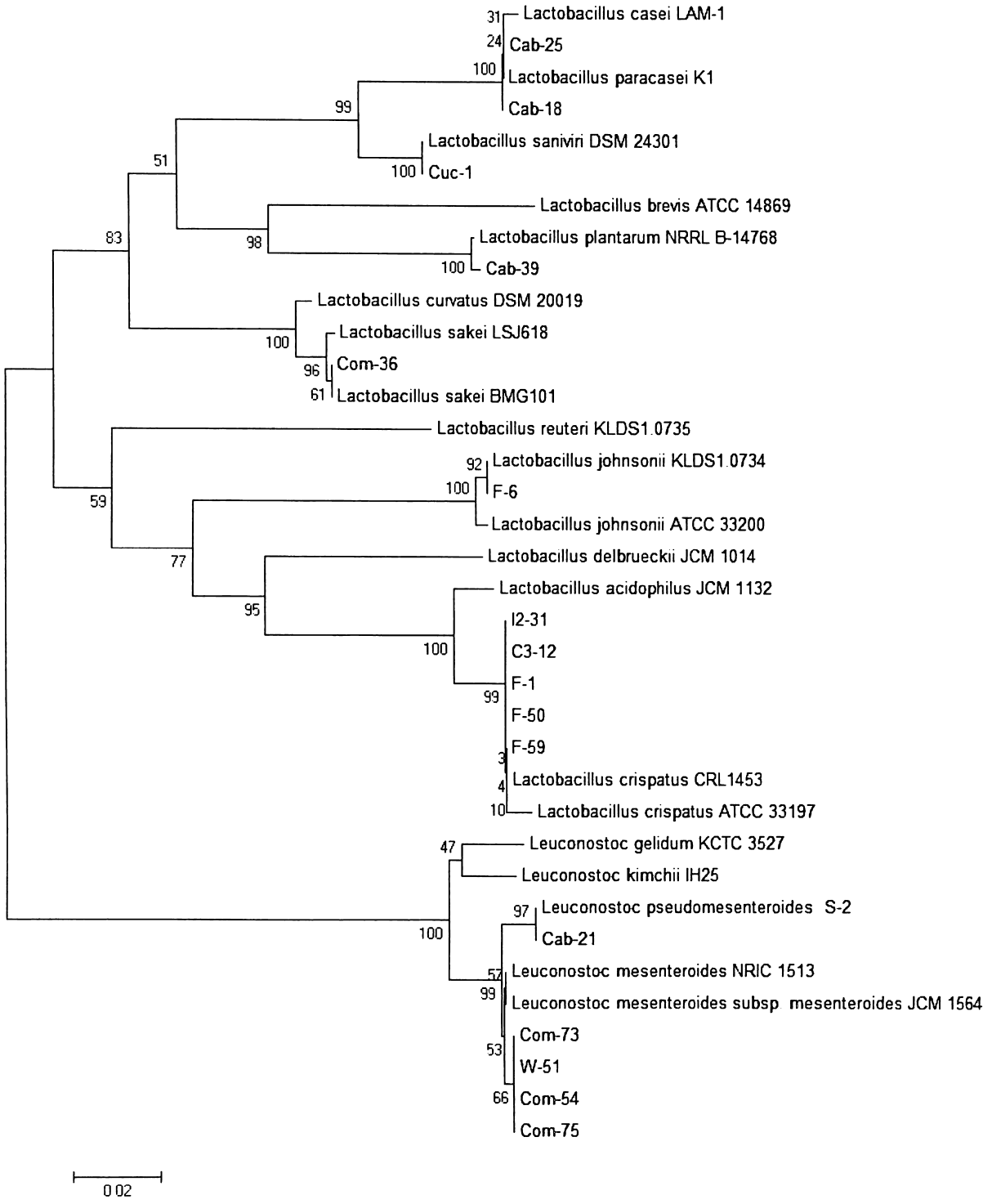


Figure 8. Neighbor-joining trees from 16S rDNA sequencing of LAB isolates.

Research Conclusions

The use of the agar well diffusion allowed for the screening of all LAB isolated in this study against four *Salmonella* serotypes. The isolates whose crude bacteriocin prep (adjusted to pH 6.5) inhibited the growth of one or more of those *Salmonella* were further characterized for their probiotic abilities. After this series of probiotic characterization experiments a total of 5 isolates from this study have the potential to be safely and effectively used as probiotics. Three were isolated from Kimchi and 2 from broiler intestines. The isolates Cab-18, Cuc-1, Com-54, F-6, and F-59 produce bacteriocin and can potentially survive throughout the gastrointestinal tract and therefore can be used as probiotics. They have been identified as *Lb. casei, Lb. saniviri, Leu. mesenteroides, Lb. johnsonii,* and *Lb. crispatus* respectively. These isolates showed strong bacteriocin activity as well as tolerance of gastric juice, bile, and enzymes as well as no signs of antibiotic resistance. These isolates can be studied further for use as probiotics in humans and in broiler chicken to reduce the incidence of foodborne illness especially as it relates to *Salmonella.* Further studies should include adherence to human epithelial cells and aggregation experiments to assure that the LAB will be able to successfully colonize in the gut.

To further study bacteriocin, the crude preparations should be purified using an ammonium sulfate precipitation and also treated with enzymes such as trypin and proteinase K, which should destroy the bacteriocins and thus eliminate the inhibitory

effect seen against *Salmonella.* After purification, the amount of protein recovered can be determined by using the Bradford method for protein quantification Also, additional studies should test heat tolerance of the bacteriocins to ensure that any high temperatures, such as in cooking, will not destroy their structure and/or eliminate their antimicrobial effect. These bacteriocins should be further characterized and identified by peptide sequencing. In addition to these *in vitro* studies, it would also be necessary to test the bacteria *in vivo* to further prove their function and inhibitory properties.

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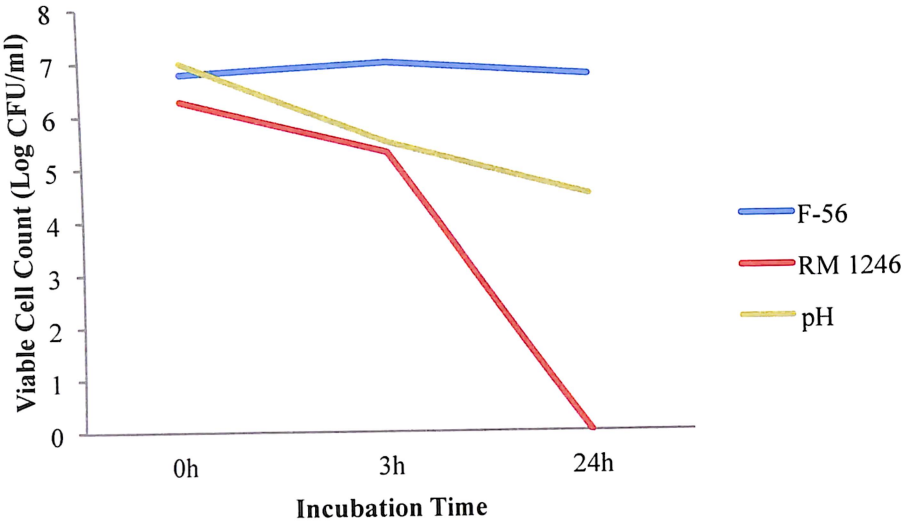
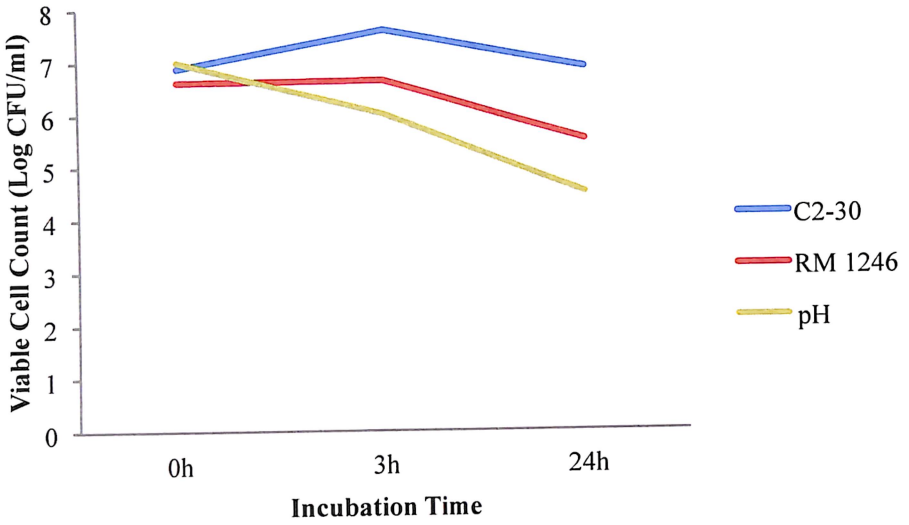
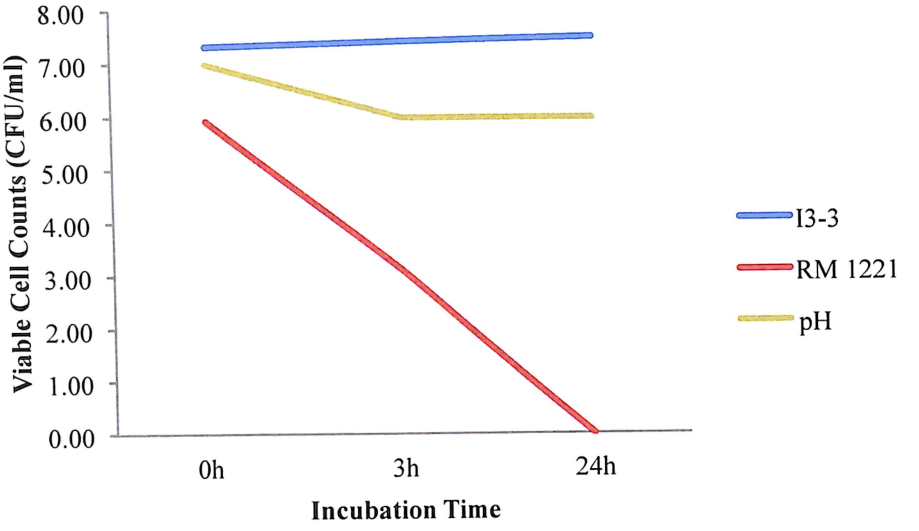
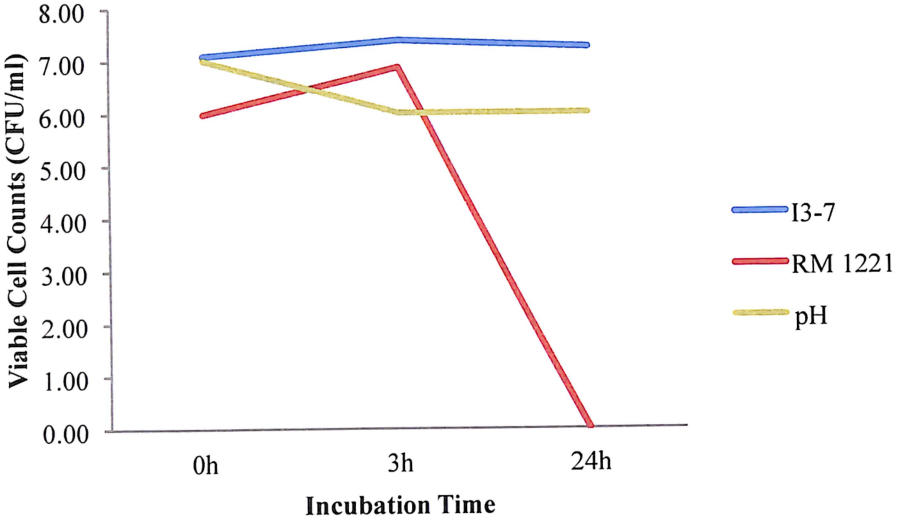
Appendices





Effect of coculture of LAB strains and *Campylobacter jejuni* RM 1246. LAB strains F-56 and C2-30 inhibited the growth of RM 1246 while in broth coculture over 24 h





Effect of coculture of LAB strains and *Campylobacter jejuni* RM 1221. LAB strains I3-3 and I3-7 and inhibited the growth of RM 1221 while in broth coculture over 24 h

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Delaware State University (DSU), Dover, DE.
May '12
Bachelor of Science in Food and Nutritional Sciences
Summa Cum Laude Latin Honor
Cumulative GPA 3.87 / 4.00

Accomplishments

- Thompson Hospitality foodservice company intern
- Study Abroad Namibia (Summer 2011)
- Department Scholar, DSU Dept. of Human Ecology, Spring `10
- President's List Fall '11, Spring '10, Fall '09; Dean's List Spring '11, Fall '10, Spring `09
- Kappa Omicron Nu Honor Society Vice President
- Minorities in Agriculture and Natural Resources and Related Sciences (MANRRS) member

Research Experience:

Delaware State University, Food Microbiology Lab
July '12 – May '13

- Isolation of bacteriocin-producing LAB from Kimchi
- Isolation of bacteriocin-producing LAB from broiler chicken
- Probiotic characterization of LAB

USDA-ARS-ERRC, MCFP Lab
May '13 – Sept. '13; May '14- Sept. '14

- LAB antimicrobial effect against *Campylobacter* (agar diffusion)
- LAB antimicrobial effect against *Campylobacter* (co-culture)

Publications and Professional Attendance:

- Inhibition of *Salmonella* by Bacteriocin-Producing Lactic Acid Bacteria Derived from U.S. Kimchi and Broiler Chicken. *Journal of Food Safety.* 2014
- Screening and Probiotic Characterization of Bacteriocin-Producing Lactic Acid Bacteria Isolated from Broilers and Kimchi. Association of Research Directors, Inc. (ARD). Graduate Student Competitive Oral Presentation. Category of "Food Safety, Nutrition, and Health" Second Place. 2013

Professional Experience:

Delaware State University, Department of Human Ecology, Dover, DE
Supervisor, Dr. Samuel Besong, Chairperson. (302) 857-6440

Student Assistant
Jan. '11 – May '12

- Organized and assisted with maintaining approximately 50 student files
- Provided responses for daily departmental inquiries of students, faculty, staff, and the public
- Assisted with basic secretarial duties such as typing, faxing, copying, and organizing office correspondence

Office of the State Superintendent of Education, Washington, D.C.
Supervisor, Christi Dorsey, RD. (202) 654-6116

Program Monitor
July '10 – Aug. '10

- Entered data of participant applications
- Organized and maintained program documents
- Reviewed and respond to incoming emails

Office Assistant
June '09 – Aug. '09

- Evaluated nutritional content of meals based on office guidelines
- Utilized e-mail system to communicate with staff, organized and maintained a variety of office files and created newsletter layout
- Developed and maintained quality control of office environment and worked on a team to host weekly workshops