# CHARACTERIZATION AND USE OF BACTERIOCIN-CONTAINING MICROBIAL FERMENTATES FOR CONTROL OF *LISTERIA MONOCYTOGENES* IN RTE MEAT APPLICATIONS

By

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CHARACTERIZATION AND USE OF GREEN-LABEL BACTERIOCIN-CONTAINING MICROBIAL FERMENTATES FOR CONTROL OF *LISTERIA MONOCYTOGENES* IN RTE MEAT APPLICATIONS

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Abstract:

Bacteriocin produced by lactic acid bacteria (LAB) are able to inhibit the growth of *Listeria monocytogenes*, and because of their GRAS (generally recognized as safe) status, they have become subjects of interest for use as additives in foods as an antimicrobial.

Bacteriocins work by different modes of action (MOA). By screening bacteriocins based on mode of action against *Listeria monocytogenes*, a multiple MOA bacteriocin cocktail can be created for use as a "hurdle" technology to inhibit the growth of *Listeria*, as well as prevent the outgrowth of spontaneously-resistant listerial strains.

We developed a series of *Listeria* strains resistant to three different MOA and screened animal-sourced samples for bacteriocins displaying a unique fourth MOA. From this procedure, two unique bacteriocin-producing bacteria were isolated and identified using 16s PCR. Cell-free supernatant (CFS) containing bacteriocin from one of these isolates, *Streptococcus* spp. 323 was purified using ammonium sulfate precipitation, C18 sep-pack elution, and elution from reverse-phase HPLC with an acetonitrile gradient. Purified sample was submitted to the Oklahoma State University Core Facility for analysis by mass spectrometry.

Certain bacteriocin-producing cultures have been implicated as opportunistic pathogens and thus merits analysis of these strains for virulence factors prior to application of CFS in foods. Streptococcal and enterococcal strains were analyzed for hemolysin and gelatinase production, with only one strain producing hemolysin and four displaying gelatinase production. Results assisted in the development of a bacteriocin cocktail utilizing 3 MOA for use in hotdog applications.

Frankfurters were formulated with different antimicrobial treatments (bacteriocin cocktail applications, NovaGard, Durafresh 2016) and underwent a series of shelf-life studies. Studies showed antimicrobials tested had a significant decrease in *L. monocytogenes* from control hotdog batches. Most notably, the use of bacteriocin cocktails within the meat matrix provided a nearly 7-log reduction 16 weeks after inoculation with *L. monocytogenes.*

Given the results of this study, the use of multiple MOA bacteriocin cocktails significantly reduce *L. monocytogenes* in hotdogs and can be used as an effective antimicrobial intervention in foods.

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## REVIEW OF LITERATURE

### INTRODUCTION

Foodborne illness due to pathogens such as *Salmonella* spp., *E. coli*, and *Listeria monocytogenes*, affects an estimated 48 million individuals each year in the United States (Centers for Disease Control and Prevention, 2016). Recent outbreaks of listeriosis, caused by *Listeria monocytogenes*, signal the urgency with which better antimicrobial solutions must be found within the food industry. An ideal solution would be an effective, natural product, solving both safety concerns and manufacturers' desires for clean labels.

Listeriosis contributes an estimated 260 deaths to the annual 3000 deaths resulting from foodborne illness. While this is a small proportion of the annual number of foodborne illnesses in the United States, the severity of this illness contributes to its high mortality rate (Centers for Disease Control and Prevention, 2017). Consumption of certain foods, including deli meats, cheeses, and frankfurters, have a high risk of the disease associated with them (Center for Food Safety & Applied Nutrition et al., 2003), and thus merit concern within the food industry.

Accompanying these safety concerns, today's consumer is increasingly conscious of their health and the products they consume. Because of this, recent trends aim toward the production of minimally processed foods without the use of chemical preservatives (Cleveland et al., 2001).

Use of bacteriocins, short antimicrobial peptides produced by lactic acid bacteria (LAB), could solve this issue, as LAB have been safely consumed for millennia in a wide variety of fermented products. The diversity of foods in which LAB bacteriocins can be found also contributes to interest in use of these products, as they are able to tolerate diverse conditions.

Previous studies have looked at the potential role of bacteriocins from LAB as antimicrobial measures in ready-to-eat (RTE) foods (Budde et al., 2003; Chen et al., 2004; Mataragas et al., 2003; Vijayakumar & Muriana, 2017). Additions of such products could help inhibit *L. monocytogenes* growth when used as a "hurdle" technology (Muriana, 1996). Such "hurdles" for *Listeria* could include pairing of bacteriocins with other antimicrobials to work synergistically, or, as proposed in this paper as well as Vijayakumar and Muriana (2017) , the use of bacteriocin cocktails working by multiple modes of action (MOA) to prevent resistant *Listeria* from overcoming the antagonistic action of a single bacteriocin. Use of this bacterially-derived solution would present a safe, natural intervention for use in industry.

***Listeria monocytogenes Virulence***

*Listeria monocytogenes* is a gram-positive pathogen responsible for approximately 1600 illnesses and 260 deaths each year (Centers for Disease Control and Prevention, 2017). Individuals susceptible to contracting listeriosis include children, the elderly, pregnant women, and other immunocompromised individuals. One specific concern is the ability of *Listeria* to cross the blood/brain barrier, as well as the placenta during pregnancy, which causes miscarriages.

While other strains of *Listeria* spp. are not considered virulent to humans, *Listeria monocytogenes* contains several virulence factors that contribute to its ability to cause disease. Interalins A and B are surface proteins that mediate entry into the host cell (de Souza Santos & Orth, 2015), while listeriolysin O (LLO) is also a virulence factor implicated in *Listeria*'s ability to cause infections, allowing the pathogen to enter the cytosol of the host cell (Jordan et al.,

2015). *Listeria* spreads intracellularly by the use of the virulence factor internalin C and is also able to manipulate actin structures within the cell to assist in movement, effectively avoiding the immune system (de Souza Santos & Orth, 2015). The virulence of this pathogen has led to *Listeria monocytogenes* outbreaks and has caused serious concern in the food industry, as contaminated food is the primary cause of listeriosis (Jordan et al., 2015).

### *Role of Listeria monocytogenes within the Food Industry*

Foodborne illness is a serious issue in the United States, as an estimated 128,000 hospitalization cases occur annually with 3000 resulting in death (Centers for Disease Control and Prevention, 2016). One important sector of these illnesses includes those caused by *Listeria monocytogenes*. Several factors contribute to the persistence of *Listeria* in the food industry. Not only is it a ubiquitous organism in the nature, but it also is salt tolerant, psychrotrophic, and establishes residence easily in food manufacturing facilities due to its ability to form biofilms (Kouakou et al., 2010; Luber et al., 2011), allowing for successful evasion of common food preservation and food safety practices. Biofilm formation, in particular, allows for the persistence of the pathogen in food production environments as it contributes to the ability of *Listeria* to tolerate industrial sanitizers by providing a protective layer. Niches of biofilms are established in areas where equipment is difficult to clean and reach, as well as within grooves and scratches in worn equipment (Jordan et al., 2015).

With outbreaks in products such as Blue Bell ice cream and cantaloupes (Center for Disease Control and Prevention, 2018a), it is apparent that steps taken to prevent contamination in industry are not always effective. *Listeria* is particularly problematic in the ready-to-eat (RTE) sector of the food industry, where cooking products is not required prior to ingestion by the consumer. Because of the dangers of listeriosis, RTE foods have been labeled as “high risk” (Center for Food Safety & Applied Nutrition et al., 2003; Rocourt et al., 2003). In an evaluation

of 20 different brands of hotdogs, one of the major at-risk foods, Wang and Muriana (1994) identified *L. monocytogenes* in 6 of the brands tested, a 30% incidence rate. A similar study by Ahmed et al. (2015) was recently conducted well after the implementation of HACCP in the food industry found an incidence rate of 2.07% in the 1883 RTE meat samples tested. While the frequency of *Listeria* illnesses has decreased since 2002 (Painter, 2013), outbreaks of this pathogen result in a higher percentage of hospitalizations than pathogens such as *Salmonella* and *Clostridium perfringens* (Centers for Disease Control and Prevention, 2016). This severity of listeriosis contributed to the decision to implement "zero tolerance" for this pathogen in foods in the United States (Gombas et al., 2003).

The "zero tolerance" policy has given rise to regulations by the United States Department of Agriculture's Food Safety and Inspection Service (Food Safety and Inspection Service, 2003). These indicate three different alternatives for implementation in RTE foods regarding the control of *L. monocytogenes*. Alternative I requires the use of a post-processing treatment as well as an antimicrobial, Alternative II involves the use of either a post-processing treatment or application of an antimicrobial, and Alternative III utilizes no additional post-processing measures, but relies only on GMPs (Good Manufacturing Practices), plant sanitation, and HACCP plans for sufficient food safety. Processors employing Alternative III as a strategy, of course, are subject to a greater amount of review by regulatory agencies, due to the higher risk involved (Zhu et al., 2005).

Because hotdogs are considered high risk foods for contamination with *L. monocytogenes*, additions of lactate and diacetate have been allowed as one of many antimicrobial interventions in frankfurters to prevent growth of the pathogen (Maks et al., 2010); however, consumers' demand for clean labels in recent years has challenged the food industry to find effective natural solutions to combat *Listeria*. One proposed solution is the use of bacteriocins, which are short antimicrobial peptides produced by a variety of bacteria. Bacteriocins from lactic acid bacteria (LAB), which

are naturally found in a wide variety of foods, have antagonistic activity against *L. monocytogene*s, which has led to a wide variety of studies performed in this area.

### *Lactic Acid Bacteria (LAB)*

The LAB are a group of Gram-positive, microaerophilic bacteria that produce lactic acid by the fermentation of hexose sugars and do not produce catalase (Coolbear et al., 2011; Lawrence Berkeley National Laboratory et al., 2006). Along with lactic acid, these bacteria produce many other compounds, including hydrogen peroxide, organic acids, and bacteriocins (O'Sullivan et al., 2002). Several different genera of bacteria fall into the definition of LAB as traditionally used in fermentations and with food products include *Carnobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* spp. (Nettles & Barefoot, 1993), but nearly 20 different genera have also been defined as LAB (Coolbear et al., 2011). Strains of LAB have been consumed safely for thousands of years, as they are used in a variety of different foods, such as cheeses, fermented sausages, and sauerkraut. These microbes are also credited with giving specific sensory characteristics unique to certain products (Coolbear et al., 2011) in addition to the protective action against certain pathogens (McAuliffe et al., 2001).Because of their history of safe use and consumption, they have been granted GRAS (generally recognized as safe) status by the FDA, allowing their use as food additives (Coolbear et al., 2011; Perez et al., 2014).

### *LAB Bacteriocins*

Bacteriocins are short, ribosomally synthesized peptides, which show inhibitory effects, either bactericidal or bacteriostatic, towards other closely-related bacteria, including bacteria such as *L. monocytogenes*, *Staphylococcus aureus*, and *Clostridium* spp. (Klaenhammer, 1993; Nettles & Barefoot, 1993). Some bacteriocins, including nisin and other class I bacteriocins, are subject to post-translational modifications (Jack & Sahl, 1995; McAuliffe et al., 2001). Strains of LAB bacteria can encode multiple bacteriocins in their genes, though all bacteriocins may not be

expressed at once (Henning, 2016; Nes et al., 2007). In fact, production of one bacteriocin can hinder the production of others by the same bacterium (Perez et al., 2012). Bacteriocins work by several different modes of action (MOA), which include the inhibition of peptidoglycan, pore formation, or affecting the target organism's DNA or RNA (Cleveland et al., 2001).

These naturally-produced peptides are promising for the food industry due to their heat and pH stability, as well as their safe ingestion by humans. Bacteriocins can have a wide or narrow spectrum of activity, meaning a single bacteriocin could have antagonistic effects on multiple genera, or could display activity against very few bacteria. This difference in the amount of activity could be beneficial and applicable to the food industry. Wide spectrum bacteriocins could be utilized as an antimicrobial measure in foods that have high risk associated with multiple pathogens, such as vacuum-packaged RTE foods, where *C. botulinum* and *L. monocytogenes* would be pathogens of concern. On the other hand, bacteriocins with a narrow spectrum could also be beneficial for industry, as issues with compatibility of bacteriocins with other starter cultures would be more easily avoided with narrow spectra of activity.

### *Classes of Bacteriocins*

Bacteriocins are typically categorized into 4 separate classes (Klaenhammer, 1988). Class I bacteriocins consist of lantibiotics, which are composed of the unique amino acid lanthionine, and include nisin (McAuliffe et al., 2001). Class II bacteriocins, which are small heat-stable peptides, are composed of three subgroups. Class IIa bacteriocins are characterized by their anti-Listerial activity, class IIb include bacteriocins requiring two peptide components for antagonistic activity, and class IIc bacteriocins require an additional thiol component for activity. Bacteriocins displaying anti-Listerial effects commonly include those found in classes I and II. Class III bacteriocins are large (>30kDa), heat labile bacteriocins, and finally class IV bacteriocins require

additional non-proteinaceous components for antagonistic activity, such as carbohydrates or lipids (Klaenhammer, 1988; Vijayakumar, 2014) .

Both class I and class II bacteriocins display antagonistic activity towards *L. monocytogenes*, leading to their interest for use in the food industry. Class I bacteriocins include lantibiotics, such as nisin and cytolysin, an enterocin. Nisin is currently the only bacteriocin approved by the World Health Organization (WHO) for use in foods (Jones et al., 2005), though the bacteriocins can be readily isolated in foods containing LAB.

***History and Use of Nisin within the Food Industry***

Nisin is a class I bacteriocin produced by *Lactococcus lactis* (Guinane et al., 2005) and is particularly unique due to post-translational alterations, leading to the presence of a lanthionine amino acid (Jack & Sahl, 1995; McAuliffe et al., 2001). Nisin was first isolated in 1928 upon observations of inhibitory effects of certain LAB against others (McAuliffe et al., 2001). Since then, eight different nisin types have been identified, including Nisin A, F, Z, H, Q, U, U2, and P (Kaskonien et al., 2017; O'Connor et al., 2015). The lantibiotic is used in over 40 countries since being granted approval by the World Health Organization (WHO) (Bouttefroy & Millière, 2000), and given GRAS status by the USDA in 1988 (USDA, 1988; (Cleveland et al., 2001). The use of nisin as a direct food additive is allowed in food products within the United States as well, due to its historical safe use before the implementation of the 1959 Food Additives Amendment. Compounds without a history of safe use require intensive testing before they can be approved and incorporated as food additives, as mentioned in 21 CFR § 170.30 (2018).

Nisin has been used in food systems for multiple benefits. It has been shown to inhibit germination of *Clostridium* spores in foods, as well as inhibit growth of *L. monocytogenes* (Cleveland et al., 2001; Davies & Adams, 1994; Gravesen et al., 2002; McAuliffe et al., 2001). Nisin is sold as a commercial antimicrobial under the name Nisaplin® (Muriana & Kanach,

1995) and is used by companies including Sysco and Kraft Foods (Jones et al., 2005). Nisin is also used in other antimicrobial mixtures, such as NovaGard®, marketed by Danisco.

Nisin operates by the disruption of the proton motor force (PMF) in *L. monocytogenes*, causing the potassium ions to surge out of the cell (Abee et al., 1995). This disruption can be overcome. Resistant strains of nisin can result from gradual increases in exposure of a strain to nisin (Ming Xintian & Daeschel, 1993) or by mutations in the fatty acids within the target cell's membrane, leading to decreased pore formation by the bacteriocin (Schillinger et al., 1998).

Successful inhibition of *L. monocytogenes* (Aasen et al., 2003; Davies et al., 1997) and *C. botulinum* spores (Okereke & Montville, 1991) by nisin has been well documented in a number of studies. However, it has been suggested that better inhibitory activity may be seen when combining nisin with other antimicrobial measures or by pairing it with class II bacteriocins (Schillinger et al., 1998; Wan Norhana et al., 2012). One important factor that must be considered when utilizing nisin in fermented products is the utilization of nisin-resistant starter cultures when additional cultures are used (Davies et al., 1997; Harris et al., 1992). Without accounting for the compatibility of cultures with nisin in the product manufactured, the fermentation process could fail due to the inhibition of the other cultures by nisin.

### *Class IIa Bacteriocins*

Class IIa bacteriocins are of particular interest in the food industry due to the subclass's noted ability to inhibit *Listeria*. These are readily found in LAB and display a variety of activity ranges. Bacteriocins of this subclass typically contain between 37 and 48 amino acid residues, contain a high proportion of glycine residues, and contain a disulfide bridge (Ennahar et al., 2000b). Other common characteristics of class IIa include a conserved sequence YGNGV, present near the N-terminal end, as well as high amounts of variation towards the C-terminus (Ennahar et al., 2000b; McAuliffe et al., 2001).

Class IIa bacteriocins target a variety of related organisms (Ennahar et al., 1999; Vijayakumar, 2014). The amphiphilic nature of these molecules allows them to bind electrostatically to a cell's wall, and then operate by forming a pore in a target cell's membrane, disrupting the balance of ions and allowing contents within the cell to escape (Ennahar et al., 2000b). Interestingly, class IIa bacteriocin-producing strains also encode protective genes, called immunity genes, that may also give them partial immunity to other class IIa bacteriocins, but not all other class IIa bacteriocins (Ennahar et al., 2000b). Target cell death is ultimately caused by the disruption of the proton motile force, causing a severe depletion of ATP and ceasing active transport (Ennahar et al., 2000b). Furthermore, studies on genes of these LAB have indicated immunity genes show very little similarity from one bacteria to another, while the genes for the bacteriocins themselves are very closely related. It is because of the dissimilarity of the immunity genes that it is believed they only share the same receptors to demonstrate the same effect (Ennahar et al., 2000b). At least 14 class II bacteriocins exist and have been characterized, while pediocin PA-1, produced by *Pediococcus acidilactici*, is the most extensively characterized class II bacteriocin (Ennahar et al., 1999).

***Pediocin PA-1***

Pediocin PA-1 has been examined in many studies regarding its effectiveness as a bacteriocin (Chen et al., 1997; Díez et al., 2012; Nieto-Lozano et al., 2010) and in several foodstuffs using a strain of *Pediococcus acidilactici* marketed as ALTA™2341 by Quest International (Chen et al., 2004; Rodríguez et al., 2002). Studies with PA-1 have mostly shown reductions of *L. monocytogenes* in foodstuffs between 1 and 3 logs (Rodríguez et al., 2002). While reduction may be statistically significant, the level of inhibition and regrowth of *Listeria* in studies also suggests application of this product in food may benefit from combination with other components to obtain synergistic action between multiple antimicrobial measures (Pucci et al, 1988).

### *Use of Enterococcus spp. Bacteriocins*

While bacteriocins have many benefits, the production of certain other damaging compounds by certain strains of LAB and their role as opportunistic pathogens has been a source of concern. Specifically, *Enterococcus* spp. and *Streptococcus* spp. have been implicated in nosocomial infections, endocarditis, and foodborne illness. This can be attributed to antibiotic resistance development by certain strains, as well as the production of aggregation substance, gelatinase, hyaluronidase, and hemolysin, among other deleterious compounds (Jett et al., 1994). However, strains of these species, particularly *Enterococcus* spp., can also contribute for development of flavor and ripening in some cheeses without harmful effects (Franz et al., 1999).

Enterococci produce some potent bacteriocins called enterocins (Franz et al., 2007). They are commonly found in animal and human digestive tracts and typically cause no harm, but certain virulent strains have been implicated in nosocomial infections, as they are opportunistic pathogens due to certain virulence factors. Even though enterococci can be opportunistic pathogens, these are isolated to only certain strains of *E. faecalis* and *faecium* (Jett et al., 1994),

while many strains do not show virulence and are safely used in food products around the world. Members of *Enterococcus* spp. are credited for giving certain artisanal cheeses their characteristic flavors because of their ability to produce certain compounds, such as acetaldehyde (Franz et al., 1999). They have also been used as probiotics (Klein, 2003) and have been utilized to reestablish balance to intestines after microbiota disruption by diarrheal diseases (Franz et al., 2011). In order to circumvent issues with infectious strains, screening of strains for compounds related to pathogenicity has been performed and some studies have looked at the use of cell-free supernatant against *Listeria* (Barman et al., 2014; Hartmann et al., 2011). Enterococci have also been implicated in the spoilage of meat (Franz et al., 1999), so use of its bacteriocins as a cell-free supernatant (CFS) would address this issue, as well as the concern over certain strains'

virulence. By ensuring the absence of virulence factors from CFS, the benefits of *Enterococcus* spp. could be employed for the improvement of food safety.

***Bacteriocin Production***

Bacteriocin production depends on a variety of factors, which include carbon sources, nitrogen, pH, and incubation temperature. Media choice is vital for production of a significant amount of bacteriocin. Typically, LAB cultures are grown in MRS (De Man, Rogosa, and Sharpe) medium, but several studies have utilized M17 medium successfully (Cheigh et al., 2002; Espeche et al., 2014; Hartmann et al., 2011). MRS medium includes Tween 80, which is used as a source of fatty acids and has been found to increase bacteriocin production (Espeche et al., 2014; Todorov & Dicks, 2009).

Several studies have made adjustments to these broths to determine characteristics vital for optimum bacteriocin production. Parente and Ricciardi (1999) found both class I and some class IIa bacteriocins were produced in larger amounts when glucose was used as the carbon source; however, findings also indicated enterocin 1146 production improved with the use of sucrose. Cheigh et al. (2002) tested the effect of many different carbon sources on bacteriocin production by a strain of *Lactococcus lactis* in M17 broth, including glucose, lactose, sucrose, xylose, fructose, galactose, arabinose, and raffinose. Utilization of lactose as a carbon source was most successful in this study. While carbon source is important for bacteriocin production, the limiting factor for bacteriocin production appears to be organic nitrogen sources. Kim (1997) found nisin concentration has a positive correlation with organic nitrogen concentration in media. The previously mentioned study by Cheigh et al. (2002) also studied variations in nitrogen source, finding the use of yeast extract to improve bacteriocin production over the other sources.

Nisin production occurs throughout growth, but spikes late in the log phase of growth (Cheigh et al., 2002). Class IIa bacteriocins, on the other hand, are secreted throughout bacterial growth.

During the growth period, pH levels between 5.5 and 6.0 result in the highest level of bacteriocin production when bacteria is incubated at ideal temperatures (Cheigh et al., 2002; Parente & Ricciardi, 1999). Certain other compounds can enhance the production of bacteriocins as well. Some examples include the use of 1% ethanol, which may assist in the expression of the gene, and the use of Tween 80. However, the possibility exists that the use of Tween may prevent bacteriocin from adhering to the surface of the container in which it is produced (Parente & Ricciardi, 1999).

### *Bacteriocin Isolation and Activity Determination*

Bacteriocin-producing LAB are quite frequent in foods. These can easily be isolated from meats, cheeses, and vegetables and screened for bacteriocin production. Because of their potential as antimicrobials in foods, it is only reasonable to source LAB from foods, as they are acclimated to the environment. Several different methods have been used to isolate bacteriocin-producing LAB, but the use of an indicator strain to observe antagonistic activity by the LAB is well-established and used (Barefoot & Klaenhammer, 1983; Moraes et al., 2010; Settanni & Corsetti, 2008; Vijayakumar, 2014). After isolation of a bacteriocin-producing colony, several methods can be used to determine bacteriocin production. Determination of bacteriocin activity can be performed by a “spot-on-lawn” assay. This requires a lawn of the indicator organism, spotted with a titer of the bacteriocin on the surface after drying. After incubation, activity units (AU) of the specific bacteriocin can be determined from the last dilution made that still displays antimicrobial activity. A similar procedure is the well diffusion method, which also utilizes antagonistic activity by adding bacteriocin to a well formed in agar. (Barefoot & Klaenhammer, 1983; Schillinger & Lücke, 1989). This method relies on the distance inhibition is seen within the media against the indicator strain. (Rodríguez et al., 2002). A final method is to screen samples for bacteriocin genes against a database (Henning et al., 2015a; Knoll et al., 2008). All methods have their benefits and drawbacks. While screening for bacteriocin genes gives comprehensive information,

not all bacteriocins whose genes are identified may be expressed, and this method is typically more involved and expensive than others. The drawback with the "spot-on-lawn" method is that other inhibitory components produced by the bacteria can falsely lead to the conclusion bacteriocin activity is present. Inhibition by hydrogen peroxide, bacteriophages, and lactic acid can lead to such assumptions, but proper controls can determine their involvement (Moraes et al., 2010; Vijayakumar, 2014).

### *Resistance of Listeria to Bacteriocins*

Strains of *Listeria monocytogenes* can develop resistance to bacteriocins naturally, which is a subject of concern if bacteriocins are to be used as natural antimicrobials in foods (Ennahar et al., 2000a). While class I bacteriocins display activity against *Listeria*, resistant colonies have been shown to occur at a frequency of between $10^{-5}$ and $10^{-8}$ (Bouttefroy & Millière, 2000). In fact, resistance is estimated to occur in 1 to 8% of wild *Listeria* strains (Gravesen et al., 2002). Microorganisms can incur resistance by cutting the bacteriocin peptide, or by utilizing efflux pumps, but in *L. monocytogenes*, spontaneous resistance can also occur by gene mutations, resulting in adjustment of the fatty acid content in the cell membrane. By changing the fatty acid composition, bacteriocins have less affinity for the new molecule and therefore do not bind successfully to the membrane of the target cell; (Crandall & Montville, 1998; Maisnier-Patin & Richard, 1996). This change is evident with the development of nisin resistance by *Listeria*, as resistance occurs more frequently with changes in the cell membrane. Evidence of this includes decreased flexibility and negative charge of the *Listeria* membrane, caused by modifications in the cell membrane (Bouttefroy & Millière, 2000). Interestingly, as mentioned by Bouttefroy and Millière (2000), at low temperatures of 10°C, a 2% concentration of salt seemed to inhibit the antagonistic effect of nisin against *Listeria* (2000). Gravesen et al. (2002) mentions nisin resistance occurs gradually with increasing concentrations and suggests that resistance to the class IIa bacteriocin, pediocin, occurs naturally in certain *Listeria* strains. While this is concerning, it

has also been shown that growth of *Listeria* strains in the absence of bacteriocins can result in loss of resistance after several generations (Dykes & Hastings, 1998). In spite of the issues presented by the formation of resistant strains, Gravesen et al. (2002) found that the lag phase growth period increased and growth rate decreased for resistant strains tested, suggesting something less than complete resistance. One important point also worth noting is resistance to one bacteriocin does not mean a strain of *Listeria* is resistant to all bacteriocins, but rather, as discussed by Macwana and Muriana (2011), this indicates the strain is resistant to bacteriocins specifically operating by that mode of action.

### *Problems with Bacteriocins in Food Applications*

Application of the actual bacteriocins into foods can also present complications. When bacteriocin is incorporated into a food matrix, its amphiphilic nature becomes problematic, and it binds to food constituents, leaving less bacteriocin free to bind with *L. monocytogenes*. Another issue has presented itself regarding the use of bacteriocin-producing bacteria as one component of multi-strain starter cultures. Some starter cultures may not be resistant to the bacteriocin or the bacteriocin-producing strain added, and therefore may affect the flavor of the final product. Also, by using live bacteriocin-producing cultures alongside other starter cultures, the levels of bacteriocin-producing cultures may not reach levels high enough to achieve a significant reduction in *Listeria*.

### *Studies with Listeria Inhibition by Bacteriocins*

Because LAB are found naturally in foods, the use of bacteriocins as protective cultures or cell-free supernatants (CFS) has been the focus of food research in recent years, particularly in meats and cheeses. It has been found that in cooked or pasteurized products the lack of background bacteria allows for an opportunistic environment for *L. monocytogenes*, should the food become contaminated (Gombas et al., 2003). By applying cultures which produce bacteriocins, a

protective antimicrobial effect may be achieved. In a study on sausages by Budde et al. (2003), the use of a protective culture of *Leuconostoc carnosum* 4010 resulted in a level of *Listeria* less than 10 CFU/g in sausage slices after 28 days, in comparison with the control, which yielded 108 CFU of *L. monocytogenes* at the end of the sampling period.

In another study, the use of bacteriocin from an *Enterococcus* spp. culture paired with nisin successfully inhibited growth of *Listeria* in ham, though sodium lactate was also applied, so the pure effect of the bacteriocin was not quantified (Du et al., 2017). An assay in raw pork showed use of LAB cultures lowered *L. monocytogenes* counts for several weeks, followed by return to normal growth (Kouakou et al., 2010). This issue is mirrored in several other studies (Bouttefroy & Millière, 2000; Murray & Richard, 1997).

Addition of CFS has been a subject of research as well. By adding CFS, the issue the questionable safety of bacteriocin-producing strains is less of a concern, however the absence of culture may not contribute to a product's preferred flavor profile. Many studies have looked at the use of CFS, including one focusing on the use of the bacteriocins on pork shoulder cuts. After dip inoculation with approximately $10^7$ CFU/mL *Listeria* solution, counts were reduced for two days before growth resumed with comparable rates to the control (Murray & Richard, 1997). While results seem undesirable, levels of $10^7$ CFU/mL are not levels typically observed when products are contaminated in industry, which are typically no higher than $10^3$ CFU/g (Gnanou Besse et al., 2008). When observing the effect of CFS in various food models, Hartmann et al. (2011) found Sakacin X bacteriocin better inhibited *L. monocytogenes* growth in milk, while Sakacin A better inhibited the pathogen in ground beef models. This stresses the importance of testing bacteriocins in the foods to which they are to be applied prior to commercial application, as results vary and certain bacteriocins are better accommodated to certain food matrices for the best protection levels.

Of course, for prevention of *Listeria* growth, the addition of LAB or CFS in addition to other measures would help improve effectiveness. This may include pairing with organic salts, such as sodium lactate and diacetate, which are currently used in industry (Maks et al., 2010; Wan Norhana et al., 2012). Nisin has also been shown to have increased efficacy against Gram-negative bacteria when paired with EDTA or other chelators (Stevens et al., 1991; Wan Norhana et al., 2012).

***Use of Frankfurters as a Model for Bacteriocin Action Against Listeria monocytogenes***

Chan and Wiedmann (2008) emphasized the importance of proper storage temperatures on preventing the growth of *L. monocytogenes* in hotdogs, mentioning the lag phase time in hotdogs drastically decreases from 18 days at 4.4ºC to 6.5 days when stored at 10ºC. The ease with which the pathogen can accumulate in this product makes frankfurters an ideal environment in which to study the effect of bacteriocins against *L. monocytogenes*.

Nieto-Lozano et al. (2010) formulated a pork and beef formulation of hotdogs without any additional antimicrobials for use in comparing the effect of the CFS of a pediocin-producing strain of *Pediococcus acidilactici* against a single strain of *L. monocytogenes* at 4°C and 15°C. After being submerged in 5,000 AU CFS for 10 minutes, they were then immersed in 5.3 log CFU/mL *L. monocytogenes* CECT4031 before packaging and incubation. The study found that after 60 days incubation at 4°C, a 2-log CFU/g difference in *Listeria* between the control and the CFS-treated packages, containing 5.9 log CFU/g and 3.9 log CFU/g, respectively. At abuse temperature (15°C), the inhibition effect was not significant, as a difference of only 0.6 log CFU/g was observed.

Another study conducted by Chen et al. (2004) observed the effect of ALTA™ 2341 from Quest International, a commercial product containing pediocin, against *L. monocytogenes* in frankfurters. The beef/pork blend of hotdogs were formulated without additional antimicrobials,

and the equivalent amount of 3000 or 6000 (depending on the batch) AU of ALTA™ 2341 was applied to the hot dogs, followed by inoculation of 5.2 log CFU/g or 3.2 log CFU/g of a 5-strain blend of *L. monocytogenes*. Frankfurters were stored at 2-4°C for 14-18 hours. At low inoculation levels, results indicated a 1.5-1.8 log CFU/g reduction, while the high inoculation gave a 1.6-2.1 log CFU/g reduction.

Finally, the effect of a multiple mode of action (MOA) bacteriocin cocktail on hotdogs was analyzed by Vijayakumar and Muriana (2017). In this study, bacteriocins were separated by MOA and selected for a cocktail of CFS. Several different batches were tested, including the replacement of the water with 300 µL CFS within the hotdog formulation, CFS applied as a surface antimicrobial, and the application of CFS on casings prior to peeling. Hot dogs of each batch were treated with 100 µL of 4.0 log CFU/mL *L. monocytogenes*, prior to incubation at 5°C. After 12 weeks, frankfurters that had bacteriocin added in place of water showed a 5 log CFU/mL difference from the control, while the bacteriocin sprayed on casings prior to peeling showed an approximately 2 log CFU/mL difference, and the surface application of bacteriocin to the hotdogs gave an approximately 7 log CFU/mL difference from the control.

All these studies indicate the use of bacteriocin in frankfurters effectively inhibits the growth of *L. monocytogenes*. Further studies characterizing the effect of multiple MOA bacteriocins over the entirety of a frankfurters shelf life, analysis of different application procedures, and comparison of bacteriocins with commercial antimicrobials would establish an understanding for the practicality of their use in industry.

***Objectives of This Study***

The aim of this study was to improve the understanding of multiple MOA bacteriocin use in RTE meats. To meet this goal, several objectives were met. First, grouping of bacteriocins by MOA,

the isolation and characterization of additional bacteriocin-producing strains, and finally, the development and utilization of a multiple MOA bacteriocin cocktail in hotdogs.

The first objective was met by screening bacteriocin cultures within the laboratory collection with a strain of *L. monocytogenes*, using the process described by Macwana and Muriana (2011). This process worked by generating a strain of *L. monocytogenes* resistant to one bacteriocin, and thus cross-resistant to bacteriocins utilizing the same MOA. The process was repeated with other bacteriocins using the new resistant strain, allowing for accumulated resistance to all bacteriocins within the laboratory collection. By screening bacteriocins for MOA, the strains of LAB chosen for use in applications could be selected by bacteriocin utilization of different MOA to avoid the formation of spontaneously resistant *Listeria* strains.

To achieve the second goal, the most resistant strain of *L. monocytogenes* was used to screen animal-sourced samples for bacteria producing bacteriocins, using the method described by Henning et al. (2015b), then isolated bacteria were identified. Bacteriocins considered for utilization in a bacteriocin cocktail were screened for virulence factors, including gelatinase and hemolysin.

Finally, using information gathered about the bacteriocins and their bacteria within the laboratory collection, strains were selected to develop a multiple MOA bacteriocin cocktail. Cell-free supernatant (CFS) from each strain were combined and used in 20-week long shelf life studies against *L. monocytogenes*. Three studies in frankfurters were conducted; two comparing the effectiveness of addition of the bacteriocin cocktail developed in this study, the bacteriocin cocktail described in Vijayakumar (2014), and two commercial antimicrobials at both high and low inoculum levels. The third study analyzed various applications of bacteriocin cocktails to the hotdogs, including addition within the meat matrix and application to the casings prior to cooking.

# CHAPTER II

## CHARACTERIZATION OF LACTIC ACID BACTERIA BASED ON BACTERIOCIN MODE OF ACTION


### ABSTRACT

Bacteriocins produced by lactic acid bacteria (LAB) can inhibit the growth of *Listeria* spp. Previous work (Macwana & Muriana, 2011) identified that isolated bacteriocin-resistant strains of *Listeria monocytogenes* show cross-resistance to other bacteriocins; these bacteriocins were considered to possess the same mode-of-action (MOA). However other bacteriocins assumed to be utilizing different MOAs still inhibited the bacteriocin-resistant strain. As additional bacteriocin-resistance was incurred on top of the previously-acquired resistances with bacteriocins of a different MOA, the strain with multiple-bacteriocin-resistance served as a bacterial screen for our search of bacteriocins possessing new MOAs.

Objectives of this work included a) screening for bacteriocin-producing bacteria expressing rare MOA bacteriocins, b) analyzing non-traditional LAB for presence/absence of putative virulence factors, and c) analyzing mixtures of MOAs to obtain the best bactericidal activity against *Listeria*. Samples were screened for bacteriocins expressing antagonistic effects against *L. monocytogenes* 39-2 $R_0$ and our multiple-bacteriocin resistant strain of *L. monocytogenes* 39-2 ($R_3$), which was used to screen bacteriocin-producing LAB for bacteriocins expressing rare

MOA. Organisms producing such bacteriocins were identified using 16s rRNA PCR and sequence analysis.

Prior to application in a bacteriocin cocktail, enterococcal strains were tested for hemolysin and gelatinase activity as potential virulence factors, and mixtures of different MOA bacteriocins were analyzed for activity against *L. monocytogenes.*

From this study, 6 isolates were inhibitory to *L. monocytogenes* 39-2 $R_0$, of which only 2 (*Streptococcus* spp. 323, *Streptococcus* spp. 707) were inhibitory to *L. monocytogenes* 39-2 $R_3$, which was resistant to bacteriocins representing 3 different modes of action. These 2 bacteriocins, therefore, represent a unique ($4^{th}$) mode of action. From 20 $Bac^+$ *Enterococcus* strains tested, only four strains of *Enterococcus faecalis* (BJ-12, BJ-13, BJ-19, and BJ-27) produced gelatinase, while none of these strains displayed hemolysin activity on horse blood agar in comparison to a hemolysin-producing control strain.

Because bacteriocin resistance can occur against individual bacteriocins, later studies will address mixtures comprising multiple MOAs for use as an antimicrobial against *L. monocytogenes*.

## INTRODUCTION

*Listeria monocytogenes* is a pathogen of concern in ready-to-eat (RTE) foods and has been the cause of many recalls and illnesses, including outbreaks with cheese, ice cream, and cantaloupe (Center for Disease Control and Prevention, 2018b). *Listeria* is a ubiquitous organism and can be

found in the environment, but often gains access to food systems because of poor sanitation or post-processing contamination. For this reason, hotdogs are particularly high risk foods for *Listeria* contamination. Within the industry, measures are taken to prevent growth of the pathogen, such as the addition of lactates and diacetates; however, consumers in recent years have initiated a movement for less processed, naturally-derived products from the food industry. To comply with consumer demands, natural intervention strategies have been more greatly desired and researched.

One potential natural intervention is the use of bacteriocins from LAB as antimicrobials. Lactic acid bacteria are Generally Recognized as Safe (GRAS) due to their history of safe use in a wide variety of food products, which logically led to researching bacteriocins for use in foods as antimicrobials. A variety of studies have been conducted with these antimicrobial peptides, including research in cheese (Dal Bello et al., 2011), ham (Du et al., 2017), wine (Díez et al., 2012), and hotdogs (Chen et al., 2004; Vijayakumar & Muriana, 2017). A variety of different bacteriocin application procedures have been examined, from direct addition of the bacteriocin-producing cultures to the addition of cell-free supernatant (CFS) and purified bacteriocin.

Nisin and pediocin have been used in commercial products, Nisaplin® and ALTA 2341™, respectively. While these both display antagonistic effects against *L. monocytogenes*, the pathogen also can develop spontaneous resistance. To best avoid this issue from occurring, combining bacteriocins employing different MOAs creates additional hurdles for *Listeria* to overcome, and makes resistant strains less likely to occur. Another solution would be pairing bacteriocins with another antimicrobial intervention, also creating another hurdle for *Listeria* to overcome.

The use of multiple MOA bacteriocin cocktails has been previously researched by Vijayakumar and Muriana (2017), but to our knowledge no studies have compared the effectiveness of a multiple MOA bacteriocin cocktail to commercially sold natural antimicrobial solutions, which would better address their potential for use within the food industry.

With this in mind, the objectives of this research were to screen bacteriocins by MOA using the method described by Macwana and Muriana (2011), screen samples for the presence of a bacteriocin employing a unique, rare MOA using a multiple bacteriocin-resistant *L. monocytogenes* strain, identify any virulence factors that may be present in bacteriocin-containing CFS, and develop a bacteriocin cocktail for later applications in hotdog shelf-life challenge studies where the effectiveness of the cocktail will compared to that of several commercial natural antimicrobial applications.

## MATERIALS AND METHODS

### *Preparation of Cultures for Use in Studies*

Master cultures of bacteriocin-producing lactic acid bacteria (LAB) and *Listeria monocytogenes* from the Muriana culture collection were stored at -80°C in milk-based freezing medium containing 11% non-fat dry milk, 1% glucose, and 0.2% yeast extract. Working stocks of cultures were made by inoculating 9 mL of either MRS (for LAB cultures) or TSB (for *L. monocytogenes* cultures) broths with 100-µL of culture and growing overnight for 16 hours at 30°C. These were then streaked for isolation, and a single colony was selected and grown 16 hours before preparing cultures for freezing. Cultures were centrifuged at 8000 rpm in a Sorvall® RC 5C Plus centrifuge with a SS-34 rotor for 10 minutes at 4°C, supernatant was decanted, and the remaining pellet was resuspended in the milk-based freezing medium previously described for storage at -80°C. From these working cultures, bacteria needed for studies were propagated twice overnight for 16 hours before use.

**Table 2.1.** Cultures used in this study

| Microorganism | Strain Designation | Source/Reference |
|---|---|---|
| *Listeria monocytogenes* | 39-2 $R_0$; not bacteriocin resistant | Muriana Culture Collection |
| *Listeria monocytogenes* | 39-2 $R_1$; not resistant to 56, FLS-1, 323 | This Study |
| *Listeria monocytogenes* | 39-2 $R_2$; not resistant to FLS-1, 323 | This Study |
| *Listeria monocytogenes* | 39-2 $R_3$; not resistant to 323 | This Study |
| *Lactobacillus curvatus* | FS47 | Garver and Muriana (1993) |
| *Enterococcus faecium* | FS97-2 | Henning et al. (2015a) |
| *Lactococcus lactis* | FLS-1 | Vijayakumar (2014) |
| *Enterococcus faecium* | FS56-1 | Garver and Muriana (1993) |

| | | |
|---|---|---|
| *Pediococcus acidilactici* | Bac 3 | Muriana Culture Collection |
| *Streptococcus* spp. | 707 | This Study |
| *Streptococcus* spp. | 323 | This Study |
| *Enterococcus durans* | FS707 | Henning et al. (2015b) |
| *Enterococcus faecalis* | BJ-12 | Henning et al. (2015b) |
| *Enterococcus faecalis* | BJ-13 | Henning et al. (2015b) |
| *Enterococcus faecalis* | BJ-19 | Henning et al. (2015b) |
| *Enterococcus faecalis* | BJ-27 | Henning et al. (2015b) |
| *Enterococcus faecium* | 326F | Henning et al. (2015b) |
| *Enterococcus faecium* | FS56-1 | Henning et al. (2015b) |
| *Enterococcus faecium* | FS97-2 | Henning et al. (2015b) |
| *Enterococcus faecium* | JCP B-5 | Henning et al. (2015b) |
| *Enterococcus faecium* | JCP M-2 | Henning et al. (2015b) |
| *Enterococcus faecium* | JCP-9 | Henning et al. (2015b) |
| *Enterococcus faecium* | Milk 5 | Henning et al. (2015b) |
| *Enterococcus faecium* | Milk 12 | Henning et al. (2015b) |
| *Enterococcus faecium* | NP-7 | Henning et al. (2015b) |
| *Enterococcus faecium* | Poop4 | Henning et al. (2015b) |
| *Enterococcus faecium* | THYME2 | Henning et al. (2015b) |
| *Enterococcus faecium* | THYME3 | Henning et al. (2015b) |
| *Enterococcus hirae* | 323F | Henning et al. (2015b) |
| *Enterococcus thailandicus* | FS92 | Henning et al. (2015b) |
| *Enterococcus thailandicus* | RP-1 | Henning et al. (2015b) |

**Lactic Acid Bacteria Characterization Based on Mode of Action**

***Preparation of Bacteriocin-Containing Cell Free Supernatant of Lactic Acid Bacteria Cultures***

After growth for 16 hours, bacteriocin-producing LAB cultures were centrifuged at 10,000 rpm in a Sorvall® RC 5C Plus centrifuge with a SS-34 rotor for 10 minutes, followed by the separation of the supernatant and pellet. Supernatant in volumes of 9-mL were pasteurized in a water bath at 80°C for 15 minutes to eliminate any remaining cells. Because the bacteriocins belong to heat-stable class IIa bacteriocins, pasteurization should had no effect on antagonistic activity towards *L. monocytogenes*.

***Preparation of Listeria monocytogenes 39-2 Strains for use in Bacteriocin Screening***

Using the method described by Macwana and Muriana (2011), strains of increasingly resistant *L. monocytogenes* 39-2 were generated. To accomplish this, 1-mL of cell-free supernatant (CFS) from *Lactobacillus curvatus* FS97 was applied to a plate of TSB with 1.5% agar. Full-strength *L. monocytogenes* 39-2 $R_0$ (non-resistant) culture was then spread plated on top and incubated at 30°C until resistant colonies appeared. This new FS97 (*Lb. curvatus*) resistant strain, heretofore referred to as $R_1$, was then used to screen bacteriocins within the laboratory collection for activity. Bacteriocins to which this new *L. monocytogenes* 39-2 $R_1$ was resistant, were identified as having the same MOA as the *Lactobacillus curvatus* FS97 bacteriocin.

*Listeria monocytogenes* 39-2 $R_1$ was then in turn used to create another, more compounded resistant strain. This was done by repeating the same process as before, but instead applying the CFS from *Enterococcus faecium* FS56-1, which still displayed antagonistic activity against the $R_1$ strain, to tryptic soy agar (TSA) and spread plating undiluted *L. monocytogenes* 39-2 $R_1$ onto the surface. This was again incubated at 30°C until resistant colonies appeared. This new isolate then

was labeled as *L. monocytogenes* 39-2 $R_2$ and exhibited resistance against both *Lb. curvatus* FS97 and *En. faecium* FS56-1.

After screening the $R_2$ strain of *Listeria* against bacteriocins within our collection, bacteriocin from *Lactococcus lactis* FLS-1 still displayed antagonistic activity. Using the same procedure previously used to obtain resistant *Listeria*, additional resistance was incurred to this bacteriocin to develop a strain of *Listeria monocytogenes* 39-2 $R_3$. No bacteriocins within the lab culture collection displayed resistance to the $R_3$ isolate.

***Screening of Animal-Sourced Samples for Novel Mode of Action Bacteriocins***

The previously mentioned method of separating bacteriocins based on mode of action (MOA) resulted in the development of a strain of *L. monocytogenes* 39-2 with resistance to all bacteriocins within our laboratory collection (*L. monocytogenes* 39-2 $R_1$, $R_2$, $R_3$). The *L. monocytogenes* 39-2 $R_3$ strain was used to screen bacteria from bovine samples (Guillen, 2009) for a lactic acid bacterium producing a bacteriocin operating by a different MOA.

Each animal-sourced sample was tested twice, once after enriching samples overnight for 16 hours in De Man, Rogosa, and Sharpe (MRS) Medium, and again by plating direct dilutions of the sample. The goal of screening samples without enrichment was to identify bacteria that may otherwise be outpaced by fast-growing bacteria during enrichment.

A series of ten-fold dilutions were made of each sample, using 0.1% Buffered Peptone Water (BPW) and spread plated. Then an overlay of MRS medium with 1.5% agar was applied followed

by a 24-hour incubation period. Tryptic Soy Broth (TSB) with 0.75% agar was prepared, and *L. monocytogenes* 39-2 $R_3$ was added to the mixture, resulting in a concentration of approximately $10^6$ CFU/mL. The agar was applied to the plates that had incubated whereupon the plates were subjected to another 24-hour incubation period. A large clear zone surrounding a colony tentatively indicated the inhibition of *L. monocytogenes* growth by a bacteriocin-producing bacterium. Bacterial isolates were recovered by inverting agar layers into the petri plate cover, excising agar surrounding a presumptive bacteriocin-producing colony, and isolating with a flame-sterilized loop. This process is shown in Figure 2.1, obtained from Henning (2016). To ensure the presence of bacteriocin activity, isolates were streaked for purity, and a single colony was chosen, then patch plated on two separate plates. One of the patch plates was directly overlain with TSA soft agar laced with *L. monocytogenes* at an approximate concentration of $10^6$ CFU/mL, while the other plate had no further treatment applied. Both plates incubated at 30°C overnight and presence or absence of bacteriocin activity was recorded the following day. Isolates with confirmed bacteriocin activity were collected from the untreated plate with a flame-sterilized loop, grown for 16 hours at 30°C, and prepared for frozen storage.

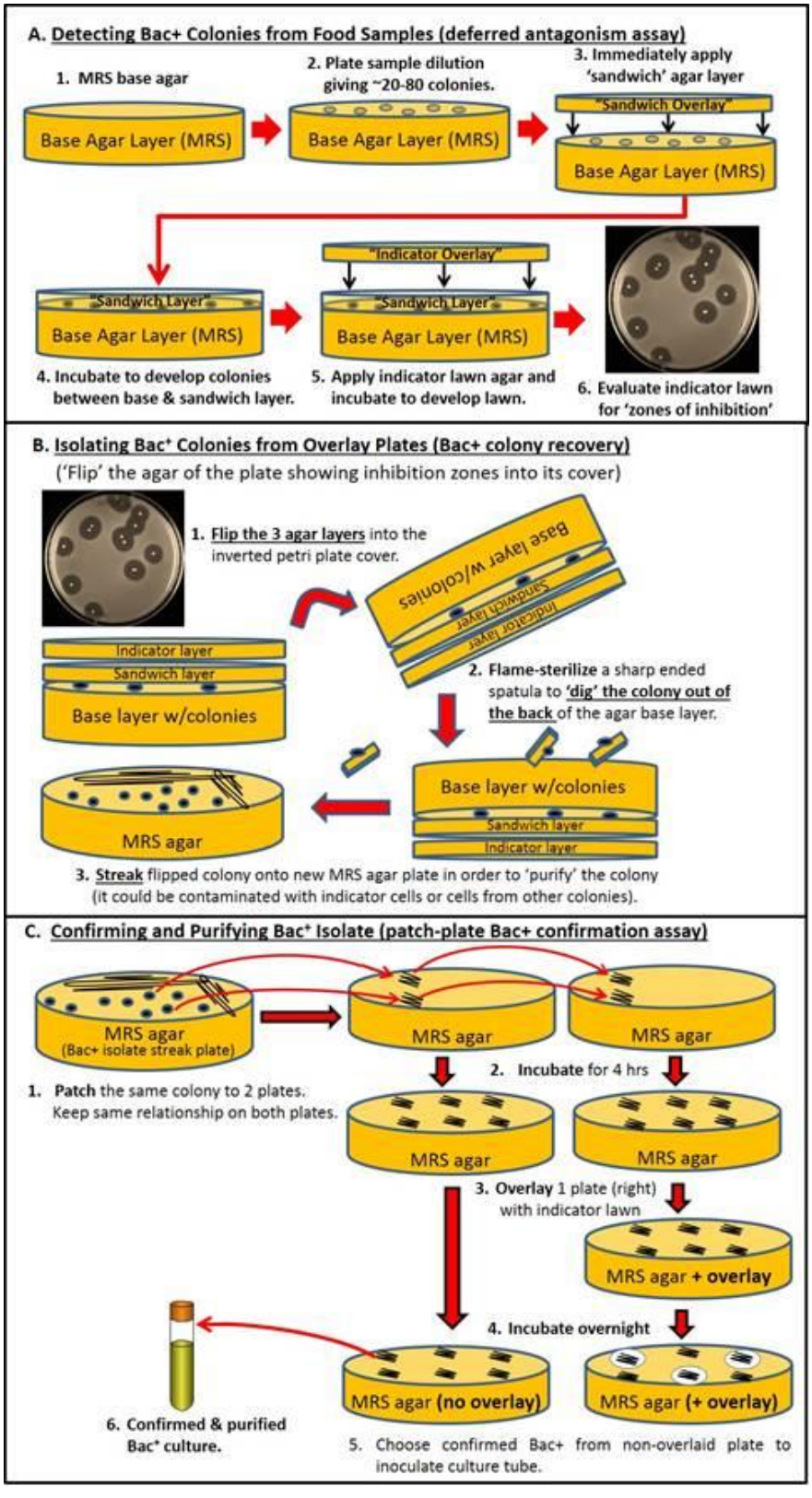
A. Detecting Bac+ Colonies from Food Samples (deferred antagonism assay)
1. MRS base agar
Base Agar Layer (MRS)
2. Plate sample dilution giving ~20-80 colonies.
Base Agar Layer (MRS)
3. Immediately apply 'sandwich' agar layer
"Sandwich Overlay"
Base Agar Layer (MRS)
"Sandwich Layer"
Base Agar Layer (MRS)
4. Incubate to develop colonies between base & sandwich layer.
"Indicator Overlay"
"Sandwich Layer"
Base Agar Layer (MRS)
5. Apply indicator lawn agar and incubate to develop lawn.
6. Evaluate indicator lawn for 'zones of inhibition'
B. Isolating Bac+ Colonies from Overlay Plates (Bac+ colony recovery)
('Flip' the agar of the plate showing inhibition zones into its cover)
1. Flip the 3 agar layers into the inverted petri plate cover.
Base layer w/colonies
Sandwich layer
Indicator layer
Indicator layer
Sandwich layer
Base layer w/colonies
2. Flame-sterilize a sharp ended spatula to 'dig' the colony out of the back of the agar base layer.
Base layer w/colonies
Sandwich layer
Indicator layer
MRS agar
3. Streak flipped colony onto new MRS agar plate in order to 'purify' the colony (it could be contaminated with indicator cells or cells from other colonies).
C. Confirming and Purifying Bac+ Isolate (patch-plate Bac+ confirmation assay)
MRS agar
(Bac+ isolate streak plate)
1. Patch the same colony to 2 plates. Keep same relationship on both plates.
MRS agar
MRS agar
2. Incubate for 4 hrs
MRS agar
MRS agar
3. Overlay 1 plate (right) with indicator lawn
MRS agar + overlay
4. Incubate overnight
MRS agar (no overlay)
MRS agar (+ overlay)
6. Confirmed & purified Bac+ culture.
5. Choose confirmed Bac+ from non-overlaid plate to inoculate culture tube.

**Figure 2.1**. Isolation of bacteriocin-producing bacteria – taken from Henning (2016).

## Isolate Identification and Characterization

### *Extraction of Bacterial DNA*

Prior to identification of bacterial isolates from screening using 16s rRNA PCR, bacterial DNA had to be extracted. First, bacteria were grown at 30°C for 16 hours before extraction. Cultures were vortexed to ensure the homogenization of the mixture, and 1.4-mL were transferred to a 1.5-mL Eppendorf microcentrifuge tube. Contents were centrifuged at 12,000xg in an Eppendorf 5424 centrifuge for 90 seconds and supernatant was discarded. A total of 500 µL of deionized water (DI) was added to the tube, and the contents were vortexed to resuspend the pellet. Samples were centrifuged again at 12,000xg for 90 seconds, before discarding the supernatant and repeating the same step with another 500-µL of DI water.

After the second wash with DI water, the supernatant was manually pipetted and discarded. This was done to avoid disrupting the pellet. Preparation of DNA was performed by the bead collision method described by Coton and Coton (2005). Depending on the size of the resulting pellet, 100-120-µL of 10 mM Tris buffer at pH 7.4 was added. After resuspension, sterile silica beads were added, then samples were subjected to several rounds alternating between vortexing and cooling on ice, intended to shear cells and expose the contents within the cell. After a final cooling period, samples were centrifuged at 12,000xg for 2 minutes. The supernatant containing the extracted DNA was then pipetted and added to another sterile microcentrifuge tube before placing the sample on ice to prevent DNAses from damaging the product.

### *Identification of Bacteriocin-Producing Isolates*

After extraction of DNA, samples could be identified using PCR. Each sample for analysis was allotted to a sterile 200-µL flat-capped PCR tube containing the components required for amplification (Table 2.3).

**Table 2.2.** PCR mix composition.

| **Component** | **Volume** |
|---|---|
| Nuclease Free DI Water | 11.75µL |
| DNA | 1µL |
| Forward Primer (515F) | 2µL |
| Reverse Primer (1391R) | 2µL |
| Taq Polymerase | 1.25µL |
| dNTP | 0.5µL |
| 5X GoTaq Flexi Buffer | 5µL |
| 25mM $MgCl_2$ | 1.5µL |
| **Total** | **25µL** |

The mixture was placed in a BioRad iCycler MyIQ™ Optics (thermal cycler), where amplification of each sample ensued alongside a positive (DNA previously extracted) and negative (water in place of DNA) control. Primers included 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 1391R (5'GACGGGCGGTGTGTRCA-3') previously

used by Henning et al. (2015). An initial denaturation at 95°C was followed by 40 cycles consisting of denaturation at 94°C for 1 minute, 58°C for 45 seconds to allow for annealing, and extension at 72°C for 1 minute. The process concluded with a final extension step at 72°C for 4 minutes, followed by cooling to 4°C.

The resulting amplification product was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin). Agarose gel electrophoresis of PCR products was performed using 1.6% (wt/vol) agarose gel with added ethidium bromide (for visualization) running at 80V on a BioRad PowerPac power supply, with a 1000-bp DNA ladder to aid in size identification of the amplimers. A BioRad ChemiDoc™ XRS transilluminator was used to view bands and verify the correct size of product, which was approximately 900-bp. A slice of the gel containing the band of DNA was excised from the gel and weighed in a 1.5-mL Eppendorf microcentrifuge tube in order to extract DNA from the gel. For purification of the DNA from this sample, the Promega Wizard® SV Gel and PCR Clean-Up System was used, beginning with the addition of the kit's membrane binding solution added in an amount of 10-µL/mg to the gel slice. Contents were vortexed and placed in a 60°C water bath until the gel had dissolved (approximately 10 minutes). The contents were again vortexed and transferred to a minicolumn, which was set into a 1.5-mL microcentrifuge tube. The melted gel solution and minicolumn were incubated at room temperature for one minute to allow for product to bind to the column. The minicolumn was then centrifuged at 12,000xg in an Eppendorf 5424 centrifuge for one minute, and contents which had passed through the column were disposed. A 700-µL aliquot of wash buffer was applied to the minicolumn and centrifuged at 12,000xg for 1 minute, then flowthrough discarded. The same step was repeated with 500-µL of wash buffer. The column was centrifuged

again, then the minicolumn was transferred to a sterile microcentrifuge tube. Nuclease-free water (50-µL) was added to the column and allowed to sit at room temperature for 1 minute to allow product to elute into the water. The column was centrifuged a final time for 1 minute at 12,000xg, then the eluate was frozen at -20°C until submission for sequencing at the Oklahoma State University Core Facility.

***Protease Assay***

Confirmation of the proteinaceous character of the bacteriocin was performed by addition of 20-µL of Pronase E (Sigma-Aldrich, St. Louis, Missouri) containing 1060-AU/mL to 80-µL of bacteriocin preparation and incubation of the mixture for 1 hour at 30°C. Loss of activity determined by a spot-on-lawn assay against *L. monocytogenes* 39-2 confirmed the proteinaceous nature of the bacteriocin.

***Catalase Assay***

Production of hydrogen peroxide by LAB occurs and can inhibit the growth of other organisms, so a catalase assay was performed to ensure hydrogen peroxide was not responsible for the observed inhibition of *L. monocytogenes.* For this assay, 20-µL of catalase (14,600 AU/mL, AMRESCO, Dallas, Texas) was applied to 80-µL of bacteriocin preparation and incubated for 1 hour at 30°C. Household hydrogen peroxide (3%) was used as a control. After a spot-on-lawn assay, loss of inhibitory activity indicated the presence of catalase.

***Bacteriocin Quantification***

Quantification of bacteriocin activity from isolates was performed by a "spot-on-lawn" assay as described by Henning et al. (2015a). Cell-free supernatant (CFS) was added to a 96 well plate and serially diluted two-fold until a series of 16 dilutions were obtained. A petri plate was scored into 8 sections with a marker and overlaid with TSA and 0.75% agar (i.e. "soft agar") containing approximately $10^6$ CFU/mL *L. monocytogenes* 39-2 $R_0$. After allowing the seeded agar media to solidify, 5-µL of the 2-fold dilutions were allocated to each section of the plate. Plates were incubated at 30°C for 24 hours, and bacteriocin strength was determined from the last dilution still showing inhibitory activity ($1/(200 \times 2^x)$, x=number of dilutions performed).

***Screening of Bacterial Isolates for Virulence Factors***

Concerns with *Streptococcus* and *Enterococcus* spp. potentially being opportunistic pathogens suggest cautious use of live strains of such bacteria in food applications should only be done after testing for the absence of potential virulence factors. Despite the fact that CFS was to be used in our work, characterization of the strains used within the bacteriocin cocktail was still be performed to alleviate any concerns. Production of hemolysin and gelatinase were tested in bacteriocin-producing *Enterococcus* spp. strains. Hemolysin was tested by the method described by Upadhyaya et al. (2010), which involved plating strains on brain heart infusion (BHI) agar supplemented with 5% horse blood and incubating at 37°C for 24 hours. Zones with clearing indicated β-hemolysis of the blood. For gelatinase production, samples were tested using the method described by Furumura et al. (2006). Strains were plated on TSA with 3% gelatin and incubated at 37°C for 24 hours. Zones of turbidity surrounding colonies indicated the presence of gelatinase.

**Purification of Bacteriocin from *Streptococcus* spp. 323.**

### *Ammonium sulfate precipitation*

The bacteriocin from the *Streptococcus* spp. 323 isolate was chosen for further characterization and identification, as the bacteriocin displayed more activity than the unique MOA bacteriocin originating from the other bacterium isolated from animal-sourced samples. After centrifuging and pasteurizing CFS from *Streptococcus* spp. 323 that had grown overnight, the *Streptococcus* spp. 323 bacteriocin was precipitated by ammonium sulfate fractionation. Ammonium sulfate was added to CFS from overnight culture to achieve a 20% saturation. This was stirred overnight at 4°C, then centrifuged at 4500 rpm in a Sorvall® RC 5C Plus centifuge with a SLA-1500 rotor for 30 minutes. The supernatant was decanted and recovered from the pellet and then brought to 30% ammonium sulfate saturation. This was again stirred overnight at 4°C before centrifuging. The resulting pellet from the 30% ammonium sulfate saturation was resuspended in 4-mL of 1X PBS buffer and subjected to a titer assay against *L. monocytogenes* 39-2 to determine the recovery level of bacteriocin activity. The remaining supernatant from the 30% ammonium sulfate fraction was then brought to 40% saturation and the same process repeated stirring this fraction overnight and centrifuging and resuspending the resulting pellet in 1X PBS buffer. The bacteriocin activity of this fraction was also determined using a titer assay.

### *C18 Sep-Pack elution*

Further purification of CFS containing bacteriocin was necessary for identification of the specific bacteriocin from *Streptococcus* spp. 323. The resuspended pellet (4-mL, 12,800 AU) from the ammonium sulfate fractions were applied to a primed C18 solid phase extraction column (BAKERBOND™ Octadecyl (C18) Disposable Extraction Columns, JT Baker, Phillipsburg, NJ). The product bound to the column was then subject to fractionation with 4-mL isopropanol using

percentages of 0% (water), 25%, 50%, 75%, and 100%. Fractions were freeze-dried, then resuspended with 1-mL sterile DI water. Resulting solutions were subjected to a two-fold dilution titer assay against *L. monocytogenes* to determine the level of activity recovered. Protein amounts present in each step of the purification process were measured with a Nanodrop ND-1000 spectrophotometer at 280-nm and bacteriocin activity measured in each fraction by a two-fold dilution titer assay.

***HPLC Chromatography***

Samples recovered from ammonium sulfate and C18 cartridge fractionation were further purified by high-performance liquid reversed-phase chromatography (HPLC) on an Agilent/HP 1050 system consisting of a quaternary pump (79852A), a diode array detector (G1306A), an autosampler (79855A), and a solvent degasser. Separation was performed on a 250 x 4.6 mm Kromasil (3.5µ) C4 column (PN 0497; Brewster, NY, USA) using a gradient of 19.25% to 81% Acetonitrile (Fisher Scientific, Raleigh, N.C.). Buffer A was made up as 5% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid (Sigma); buffer B was composed of 95% (vol/vol) acetonitrile in 0.05% trifluoroacetic acid. The gradient consisted of a 70-min linear gradient from 85% A/15% B to 20% A/80% B at a flow rate of 0.5-ml/min. Fractions were collected in 1.5-ml Eppendorf tubes in 1-mL increments every 2 minutes using a Bio-Rad fraction collector (Model 2128, Hercules, CA) and evaporated overnight on a vacuum centrifuge (Savant Instruments, Inc., Farmingdale, N.Y.), then resuspended in 100-µl of glass-distilled water. Resuspended fractions (5-µL) were spotted onto an indicator lawn of *L. monocytogenes* 39-2 $R_0$ and incubated at 30°C overnight to determine which fractions contained bacteriocin.

***Mass Spectrometry***

Fractions subjected to HPLC and showing bacteriocin activity were submitted to the Oklahoma State University Core Facility for mass spectrometry analysis.

## RESULTS AND DISCUSSION

### *Preparation of Listeria monocytogenes 39-2 Strains for use in Bacteriocin Screening*

The method of characterizing bacteriocins based on MOA as used and discussed by Macwana and Muriana (2011) was chosen for use in this study. The wild-type strain of *Listeria monocytogenes* 39-2 was sensitive to all bacteriocins to which it was exposed. These included bacteriocins produced by LAB strains *Lactobacillus curvatus* FS47, *Lactobacillus curvatus* FS97, *Pediococcus acidlactici* Bac 3, *Lactococcus lactis* FLS-1, and *Enterococcus faecium* FS56-1. When *L. monocytogenes* 39-2 spontaneously gained resistance to the *Lactobacillus curvatus* FS97 bacteriocin, resulting in *L. monocytogenes* 39-2 $R_1$, it also demonstrated cross-resistance to bacteriocins produced by *Lactobacillus curvatus* FS47 and *Pediococcus acidilactici* Bac3.

Additional resistance gained by *L. monocytogenes* 39-2 $R_1$ against the *Enterococcus faecium* FS56-1 bacteriocin, resulting in *L. monocytogenes* 39-2 $R_2$, which now demonstrated resistance to *Lb. curvatus* FS97, *Lb. curvatus* FS47, *P. acidilactici* Bac3, and *E. faecium* FS56-1. The final application of bacteriocin from *Lactococcus lactis* FLS-1 to *L. monocytogenes* 39-2 $R_2$ yielded a strain of *Listeria* ($R_3$) fully resistant to the full repertoire of bacteriocins within the lab collection, representing 3 MOA, against which the *L. monocytogenes* 39-2 based strains were tested. The *L. monocytogenes* 39-2 ($R_0$, $R_1$, $R_2$, $R_3$) isolates were used to screen for bacteriocins demonstrating a new and a unique MOA.

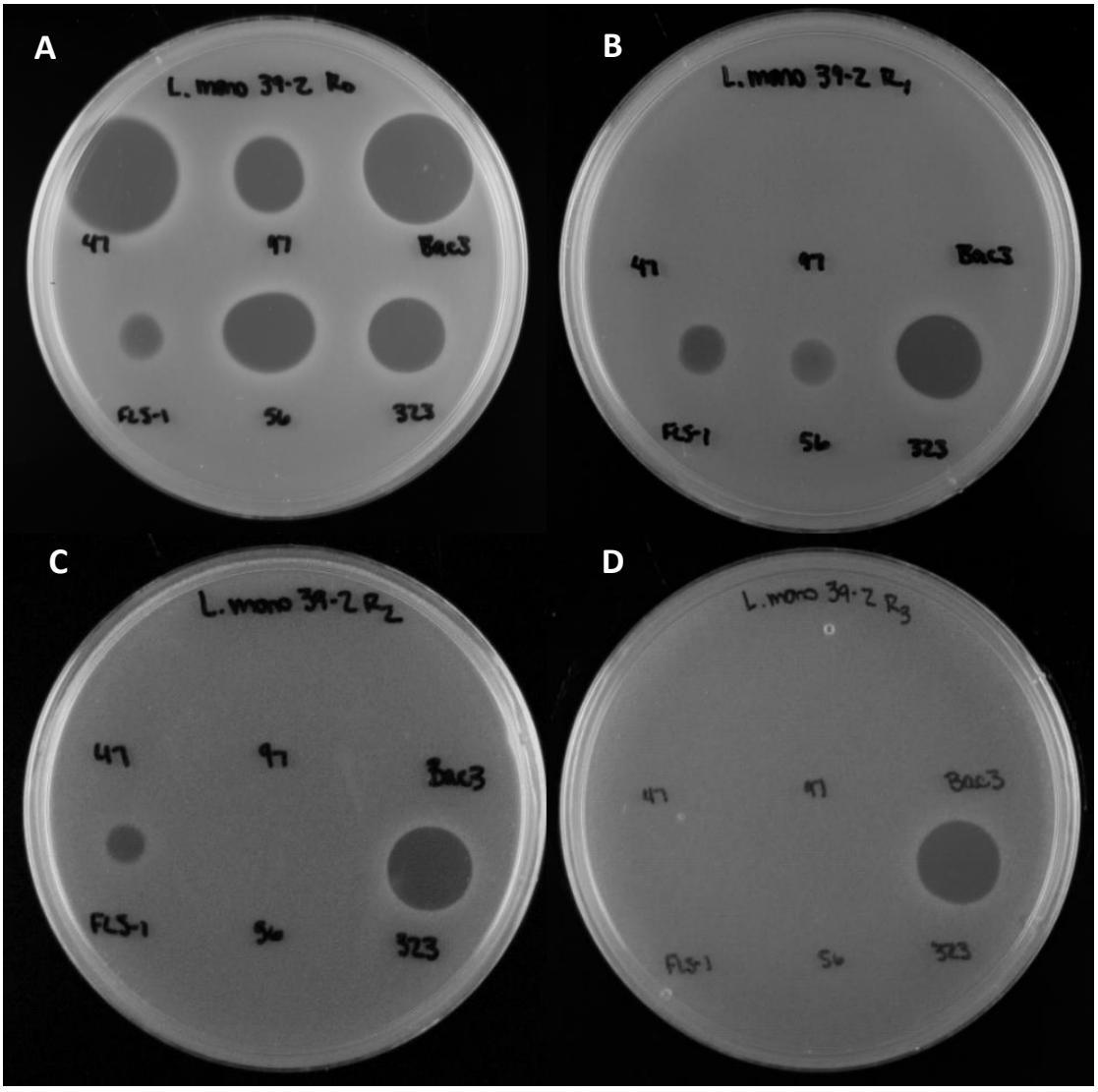


**Figure 2.2.** Series of bacteriocin-resistant variants of *Listeria monocytogenes* 39-2. Bacteriocin-containing cell-free supernatants (*Lactobacillus curvatus* FS47, *Lactobacillus curvatus* FS97, *Pediococcus acidlactici* Bac 3, *Lactococcus lactis* FLS-1, *Enterococcus faecium* FS56-1, and *Streptococcus* spp. 323) spotted on non-resistant *L. monocytogenes* $R_0$ (Panel A), *L.*

*monocytogenes* $R_1$ (Panel B), *L. monocytogenes* $R_2$ (Panel C), and *L. monocytogenes* $R_3$ (Panel D).

***Screening of Animal-Sourced Samples Utilizing Novel Mode of Action Bacteriocins***

Formation of the series of *Listeria monocytogenes* 39-2 strains ($R_0$, $R_1$, $R_2$, $R_3$) allowed for screening of samples in search of a bacteriocin displaying a specific MOA and ultimately allowed the construction of a mixture of bacteriocins displaying multiple MOAs. Samples from cattle yielded 6 total bacteriocin-producing isolates with 2 isolates operating by a unique MOA, which were detected using *L. monocytogenes* 39-2 $R_3$. These two isolates were labeled as 323 and 707, due to the names of the samples from which they were isolated, as well as AB1, AB2, AB3, and AB4. Samples screened for a novel MOA are listed below in Table 3.1. Isolation of bacteria demonstrating the rare MOA inhibitory to *L. monocytogenes* 39-2 $R_3$ were only isolated by direct plating without enrichment suggesting a slower growth rate by these bacteria, which may be overwhelmed by other LAB within the samples during enrichment.

Originally, samples were enriched for the screening process. After seeing a lack of bacteriocin-producing isolates utilizing the MOA for which the samples were being screened, the decision was made to screen samples again without enrichment in the MRS broth. The use of enrichment would give quickly-growing bacteria an edge over slower-growing bacteria. This was also discussed by Henning (2016), who observed a higher number of *Lactococcus lactis* isolates when screening samples for bacteriocins using enrichment than what was observed when no enrichment was used.

**Table 2.3.** Samples screened for novel MOA bacteriocin production with *Listeria monocytogenes* 39-2

| Animal Sample Name | Sample Enrichment | Resistant *Listeria* Used | Bacteriocin Isolated | Isolate Identified |
|---|---|---|---|---|
| 326 S+L Rum Inf Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 S+L Rum Inf Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 707 Rumen Solid DFM Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 323 S+L AbInf Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 707 DFM S+L Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 323 Rumen Solid AbInf Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 S+L Con Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 326 RumInf Rumen Solid Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 Con Rumen Solid Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 707 DFM Rumen Liquid Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 Con Rumen Liquid Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 323 AbInf Fecal Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 Con Fecal Day 0 | No Enrichment | $R_3$ | No | – |

| | | | | |
|---|---|---|---|---|
| | Enriched | $R_3$ | No | – |
| 707 DFM Fecal Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 326 RumInf Rumen Liquid Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 323 AbInf Rumen Liquid Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 707 DFM Sponge Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 323 AbInf Sponge Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 326 RumInf Sponge Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 Con Sponge Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 326 RumInf Fecal Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 326 DFM Fecal Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 323 DFM Fecal Day 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 323 Con Fecal Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 AbInf Fecal Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 326 DFM Fecal Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 707 RumInf Fecal Week 0 | No Enrichment | $R_3$ | Yes | *Streptococcus* spp. 707 |

| | | | | |
|---|---|---|---|---|
| | Enriched | $R_3$ | No | – |
| 323 Con Fecal Week 0 | No Enrichment | $R_3$ | Yes | *Streptococcus* spp. 323 |
| | Enriched | $R_3$ | No | – |
| 323 Con Rumen Liquid Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 AbInf Rumen Liquid Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 326 DFM Rumen Liquid Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 707 RumInf Rumen Liquid Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 326 DFM S+L Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 707 RumInf S+L Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 AbInf S+L Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 323 Con S+L Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 AbInf Rumen Solid Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 707 RumInf Rumen Solid Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 326 DFM Rumen Solid Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 323 Con Rumen Solid Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 323 Con Sponge Week 0 | No Enrichment | $R_3$ | No | – |

| | | | | |
|---|---|---|---|---|
| | Enriched | $R_3$ | No | – |
| 326 DFM Sponge Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 707 RumInf Sponge Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 AbInf Sponge Week 0 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 323 Con Fecal Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 AbInf Fecal Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 326 DFM Fecal Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 707 RumInf Fecal Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 323 Con Rumen Liquid Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 AbInf Rumen Liquid Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 326 DFM Rumen Liquid Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 707 RumInf Rumen Liquid Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 326 DFM S+L Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 707 RumInf S+L Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 AbInf S+L Week 1 | No Enrichment | $R_3$ | No | – |

| | | | | |
|---|---|---|---|---|
| | Enriched | $R_3$ | No | – |
| 323 Con S+L Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 AbInf Rumen Solid Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 707 RumInf Rumen Solid Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 326 DFM Rumen Solid Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 323 Con Rumen Solid Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 323 Con Sponge Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 326 DFM Sponge Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 707 RumInf Sponge Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 341 AbInf Sponge Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| 707 Con Fecal Week 1 | No Enrichment | $R_3$ | No | – |
| | Enriched | $R_3$ | No | – |
| Ground Beef | Enriched | $R_0$ | Yes | *Lactococcus lactis* AB1 |
| | Enriched | $R_0$ | Yes | *Lactococcus lactis* AB2 |
| | Enriched | $R_0$ | Yes | *Lactococcus lactis* AB3 |
| | Enriched | $R_0$ | Yes | *Lactococcus lactis* AB4 |

### *Identification of Bacteriocin-Producing Isolates*

After bacteria producing a bacteriocin with the desired MOA were isolated, the cultures were grown, and DNA from the samples were extracted, amplified by PCR, purified, and sequenced. When sequence data was compared to the NCBI database, analysis of the 16s rRNA sequence data from the samples showed that both isolates found by screening with *L. monocytogenes 39-2* ($R_3$) were streptococcal strains. The closest match for both isolates were either *Streptococcus lutetiensis*, *Streptococcus infantarius,* or *Streptococcus equinis*. All three of these had 100% sequence identity for the partial 16s rRNA sequence that was identified. Interestingly, both bacterial isolates originated from different samples, but gave similar identities. This may have to do with the location the isolates originated from, as these are common bacteria in cattle. Because these isolates may belong to Group D streptococci, virulence is an important concern that should be addressed, and due to this, the two isolates were tested alongside the *Enterococcus* spp. cultures in our collection for virulence factors such as hemolysin and gelatinase. Other isolates found in ground beef were strains of *Lactococcus lactis*, which are commonly found in meats. Moving forward with the study, samples 323 and 707 were examined more closely, as their unique MOA was of interest for use in a multiple MOA bacteriocin cocktail.

### *Protease and Catalase Assays*

After a spot-on-lawn assay, bacteriocin showed loss of activity after treatment with pronase E, indicating proteinaceous nature. Treatment with catalase had no effect on the inhibition of *L. monocytogenes*, indicating the inhibition was not due to production of hydrogen peroxide (Figure 2.3).

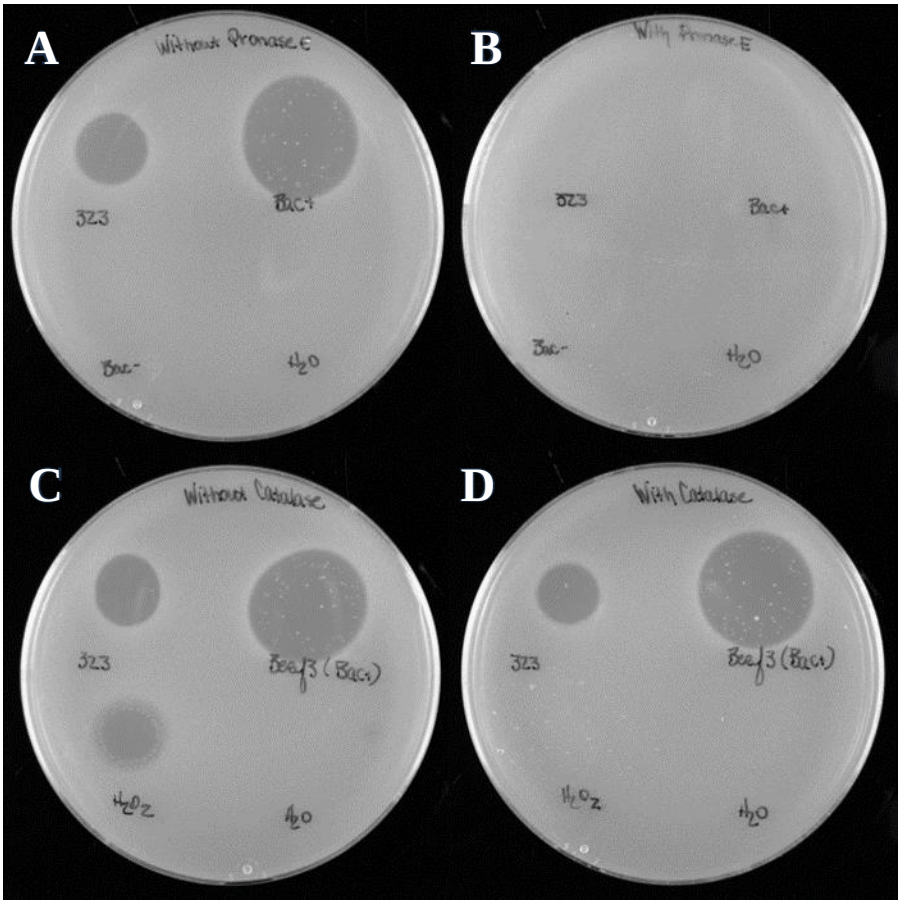


**Figure 2.3.** Bacteriocin treatments with pronase E and catalase. No treatment (Panel A) on *Streptococcus* spp. 323, *Lactobacillus curvatus* Beef 3 (Bac+), *Pediococcus acidilactici* (Bac-), and water compared to treatment with pronase E (Panel B). No treatment (Panel C) on *Streptococcus* spp. 323, *Lactobacillus curvatus* Beef 3 (Bac+), hydrogen peroxide, and water compared to treatment with catalase (Panel D).

***Determination of Bacteriocin Titer and Heat Stability***

The use of a culture supernatant as a crude bacteriocin mixture constitutes a "bacterial fermentate" and was practical for use as it was a cost-effective, simple method that could be readily applied to industrial use. However, it was vital that cells of the bacterial culture be absent

from the supernatant to avoid any potential negative effects to any product to which it would be applied, as LAB can be considered spoilage bacteria. Since the bacteriocins were resistant to heat treatment, pasteurization of the 9-mL supernatants was performed at 80°C for 10 minutes to ensure the absence of any cells. The stability of class I and II bacteriocins in response to heat has been well-established (Chen et al., 2004; Malheiros et al., 2015; Perez et al., 2014). Because the lactic acid bacteria (LAB) used in this study were heat-stable, heat pasteurization did not effect the antagonistic activity toward *L. monocytogenes*. This method was previously found to be equally as effective as filter sterilization (Vijayakumar & Muriana, 2017), which was also confirmed in this study. Heat pasteurization serves as a cheaper alternative to filtration, allowing for practical and cost-efficient application of these antimicrobials for commercial use in foods.

Analysis of each bacteriocin sample in a two-fold dilution titer assay against an indicator lawn of *Listeria monocytogenes* 39-2 $R_0$ at a concentration of approximately $10^6$ CFU/mL showed *Streptococcus* spp. 323 (3200 AU/mL) produced more bacteriocin than *Streptococcus* spp. 707 (1600 AU/mL). While samples were not neutralized to ensure antagonistic activity against was not due to acid inhibition, acid inhibition is not observed when spotting 5-µL of culture supernatants from LAB containing no bacteriocin on *Listeria* (Vijayakumar & Muriana, 2015).

***Screening of Isolates for Virulence Factors***

As previously mentioned, certain non-traditional LAB, such as enterococci and streptococci produce virulence factors that would be of concern if products containing live bacteria were to be ingested by humans. Hemolysin and gelatinase, the two virulence factors for which isolates were tested in this study, are two common virulence factors associated with these bacterial genera (Franz et al., 1999; Furumura et al., 2006; Lopes et al., 2006; Upadhyaya et al., 2010). Results of virulence tests showed only the control organism (*Enterococcus faecalis* ATCC 14508) displayed β-hemolysis on BHI with horse blood, indicated by a clearing zone around the colonies, while

four of the *Enterococcus* spp. strains tested produced gelatinase, noted by a turbid zone surrounding colonies (Figure 2.4). Researchers in literature have communicated different results when reporting positive results of gelatinase assays, obtaining both turbid halos and clear halos surrounding gelatinase-positive colonies (Furumura et al., 2006; Kanemitsu et al., 2001; Vergis et al., 2002), however the resulting zone appearance depends on the turbidity of the media itself (Smith & Goodner, 1958). These results assisted in deciding which strains to include within the bacteriocin cocktail for RTE meat applications, allowing isolates secreting questionable compounds to be avoided (Table 2.4).

**Table 2.4.** Virulence factors of non-traditional LAB isolates

| Species | Strain | Gelatinase | Hemolysin |
|---|---|---|---|
| *Enterococcus durans* | FS707 | − | − |
| *Enterococcus faecalis* | BJ-12 | + | − |
| *Enterococcus faecalis* | BJ-13 | + | − |
| *Enterococcus faecalis* | BJ-19 | + | − |
| *Enterococcus faecalis* | BJ-27 | + | − |
| *Enterococcus faecium* | 326F | − | − |
| *Enterococcus faecium* | FS56-1 | − | − |
| *Enterococcus faecium* | FS97-2 | − | − |
| *Enterococcus faecium* | JCP B-5 | − | − |
| *Enterococcus faecium* | JCP M-2 | − | − |
| *Enterococcus faecium* | JCP-9 | − | − |
| *Enterococcus faecium* | Milk12 | − | − |
| *Enterococcus faecium* | Milk5 | − | − |
| *Enterococcus faecium* | NP-7 | − | − |
| *Enterococcus faecium* | Pop4 | − | − |
| *Enterococcus faecium* | THYME2 | − | − |
| *Enterococcus faecium* | THYME3 | − | − |
| *Enterococcus hirae* | 323F | − | − |
| *Enterococcus thailandicus* | FS92 | − | − |
| *Enterococcus thailandicus* | RP-1 | − | − |
| *Enterococcus faecalis* | ATCC 19433 | − | − |
| *Enterococcus faecalis* | ATCC 14508 | − | + |
| *Streptococcus* spp. | 323 | − | − |
| *Streptococcus* spp. | 707 | − | − |

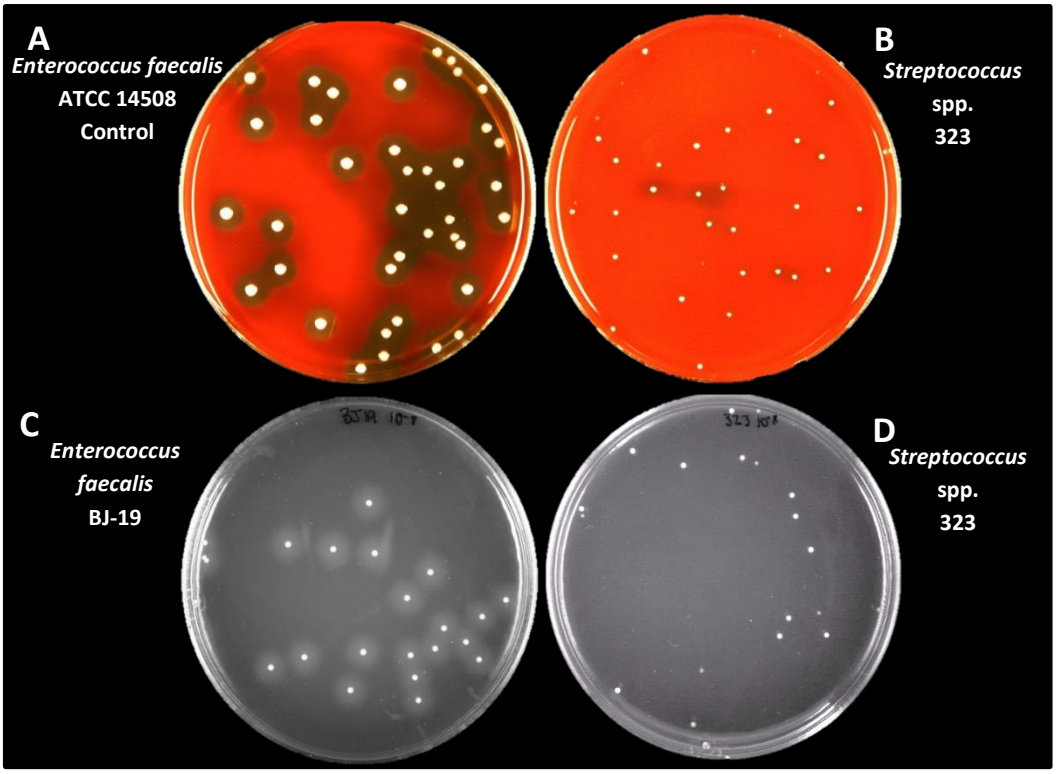


**Figure 2.4.** Positive and negative examples of virulence factors hemolysin and gelatinase. Panel A, *Enterococcus faecalis* ATCC 14508 displaying β-Hemolysis of horse blood. Panel B, *Streptococcus* spp. 323 showing absence of β-hemolysis. Panel C, *Enterococcus faecalis* BJ-19 production of gelatinase. Panel D, *Streptococcus* spp. 323 showing absence of gelatinase.

### *Purification of Bacteriocin from Streptococcus spp. 323*

Identification of the bacteriocin produced by *Streptococcus* spp. 323 would require the use of mass spectrometry, which requires the absence of detergents that cause interference when analyzing samples, such as Tween 80 (Jäpelt et al., 2016). To avoid this, M17 (no Tween) with 1% glucose was used in place of MRS for growing culture with the purpose of identifying the specific bacteriocin produced. Although *Streptococcus* spp. 323 grown in M17 broth yielded less

bacteriocin (800 AU/mL) than with MRS broth (3200 AU/mL), we chose to continue purification of bacteriocin produced in M17 broth because the absence of Tween 80 would not cause interference during mass spectrometry analyses. Despite the difference in production of the bacteriocin, mass spectrometry does not require high concentrations for identification, so purification continued using M17 broth.

Isolation began with saturation of cell-free supernatant (CFS) with ammonium sulfate in 10% increments, precipitating after each saturation level. Each supernatant recovered after centrifugation was then brought up to the next ammonium sulfate concentration in order to remove proteins precipitated at the prior level. The *Streptococcus* spp. 323 bacteriocin precipitated in the 30% and 40% ammonium sulfate fractions and were recovered after centrifugation. After resuspension of precipitated pellets from each of these fractions in 4-mL of 1X PBS, these were eluted through a C18 cartridge, followed by fractionation with isopropanol solutions at 0% (water), 25%, 50%, 75%, and 100% concentrations. The fractions containing bacteriocin eluted in the 25% and 50% fractions. These fractions were then freeze-dried and resuspended in 1-mL DI water, resulting in further purified bacteriocin solution. The fraction with the highest specific activity (i.e. bacteriocin activity units per milligram of protein) present in the sample as determined by analysis with a Nanodrop ND-1000 to determine protein concentration, was run through HPLC, and the recovered fraction showing the greatest amount of activity was submitted to the Oklahoma State University Core Facility for analysis by mass spectrometry. The protein concentration and biological activity (AU) obtained in each step throughout the process are documented below in Table 2.5.

***Mass Spectrometry***

Numerous bacteriocins are produced by streptococci known to be lantibiotics, which contain lanthionine and have many post-translational modifications. Because these contain unusual amino

acids, they are not as readily discernible by mass spectrometry protein fragmentation for identity as are traditional peptides.

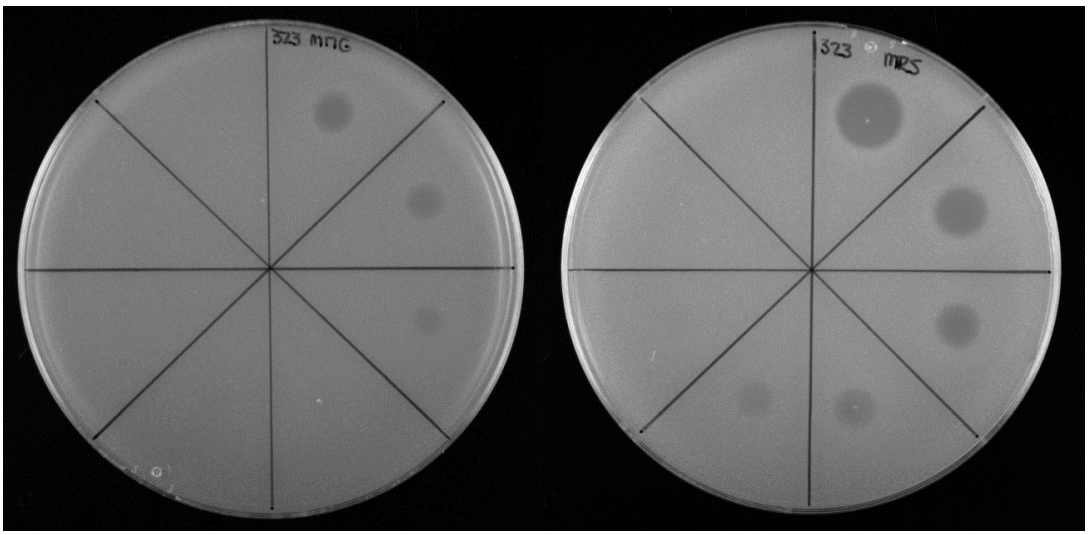


**Figure 2.5.** Comparison of *Streptococcus* spp. 323 bacteriocin production in M17G (left) and MRS (right).

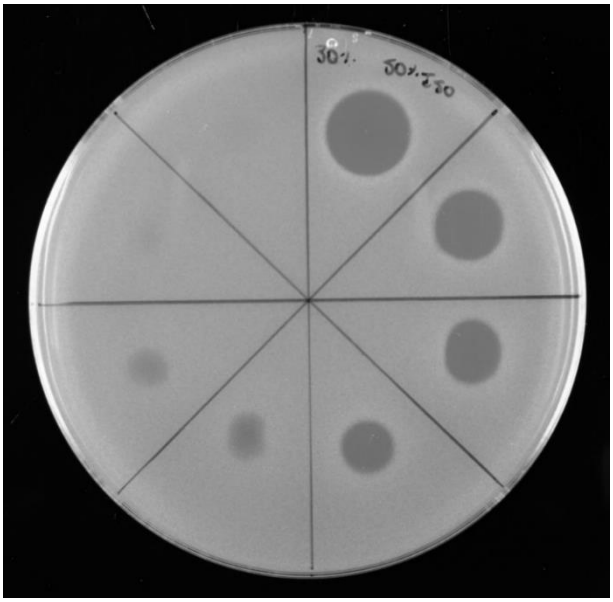


**Figure 2.6.** Activity of precipitate derived from 30% ammonium sulfate saturation fractionation followed by elution through a C18 column with 50% isopropanol.

**Table 2.5.** *Streptococcus* spp. 323 bacteriocin purification.

| Sample | Total Activity (AU) | Total Protein (mg) | Specific Activity (AU/mg) | Activity Recovered (%) | Fold Purified |
|---|---|---|---|---|---|
| Culture Supernatant (M17 Medium) | 168,000 | 4226.60 | 39.75 | 100.00% | 1 |
| Ammonium Sulfate Precipitation 30% Saturation | 51,200 | 37.53 | 909.95 | 30.48% | 22.89 |
| Sep-Pack / C18 Original Am. Sulf. 30% Eluate | 3200 | 15.65 | 204.52 | 1.90% | 5.15 |
| Sep-Pack / C18 Water Wash | 0 | 1.70 | 0 | 0.00% | 0 |
| Sep-Pack / C18 25% Fraction | 3200 | 5.08 | 629.51 | 1.90% | 15.84 |
| Sep-Pack / C18 50% Fraction | 6400 | 2.84 | 2253.52 | 3.81% | 56.69 |
| Sep-Pack / C18 75% Fraction | 0 | 0.15 | 0 | 0.00% | 0 |
| Sep-Pack / C18 100% Fraction | 0 | 0.03 | 0 | 0.00% | 0 |
| | | | | | |
| Ammonium Sulfate Precipitation 40% Saturation | 25600 | 56.27 | 349.73 | 15.24% | 8.80 |
| Sep-Pack / C18 Original Am. Sulf. 40% Eluate | 3200 | 16.98 | 188.46 | 1.90% | 4.74 |
| Sep-Pack / C18 Water Wash | 0 | 2.88 | 0 | 0.00% | 0 |
| Sep-Pack / C18 25% Fraction | 12,800 | 15.66 | 817.37 | 7.62% | 20.56 |
| Sep-Pack / C18 50% Fraction | 400 | 1.21 | 329.67 | 0.24% | 8.29 |
| Sep-Pack / C18 75% Fraction | 0 | 0.16 | 0 | 0.00% | 0 |
| Sep-Pack / C18 100% Fraction | 0 | 0.056 | 0 | 0.00% | 0 |
| HPLC Purification (RP-C4, Acetonitrile)–Fract. #12 | 320 | 0.015 | 21,333.33 | 0.19% | 536.69 |

As can be seen from the table above, the best crude purification was achieved by precipitating protein at 30% ammonium sulfate saturation, followed by elution from a C18 column with 50% isopropanol. Other eluates yielded a significant amount of bacteriocin, but this fraction yielded the best sample, as it displayed the highest specific activity (AU/mg protein). Overall, 45.72% of the bacteriocin was recovered in the ammonium sulfate fractions and after elution through the C18 cartridge, the purest fraction resulted in a 56.69-fold purification with a specific activity of 2253.52 AU/mg. During the HPLC fractionation, the bacteriocin eluted in the 12$^{th}$ fraction, which represented elutions from the 22-24 minute time period during the HPLC run. This fraction was dried and resuspended in 100-µL of water and showed a specific activity of 21,333.33 AU/mg protein, demonstrating a 536.69-fold level of purification. The HPLC step resulted in a nearly 10-fold level of recovery amplification of ammonium sulfate and sep-pack elution.

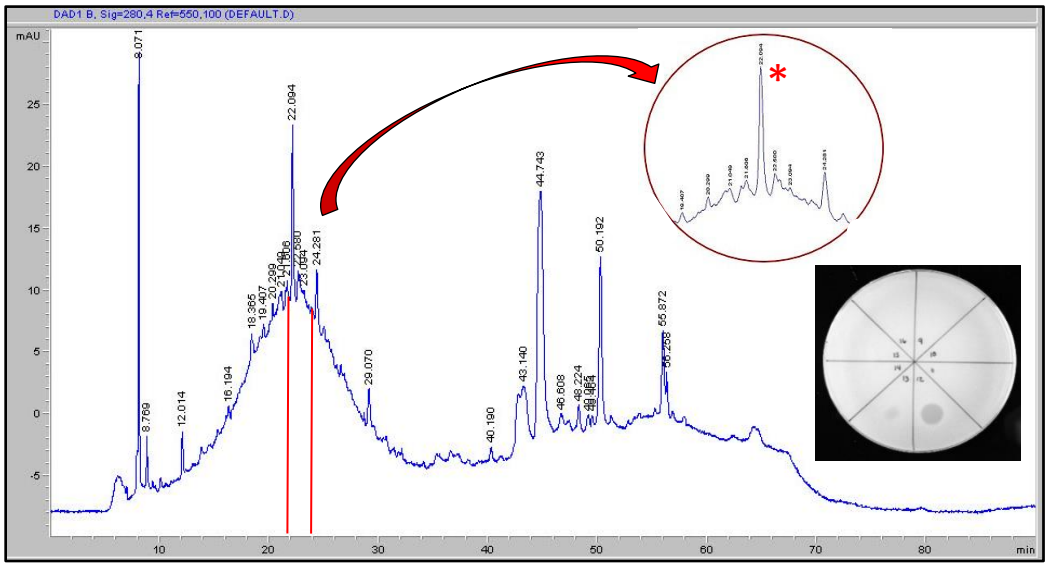


**Figure 2.7.** Fractionation using HPLC. The enlarged peak, showing detection at 280 nm, indicates the presence of the bacteriocin. When the fraction was recovered and spotted, inhibitory activity on an indicator lawn of *L. monocytogenes* 39-2 ($R_0$) was observed.

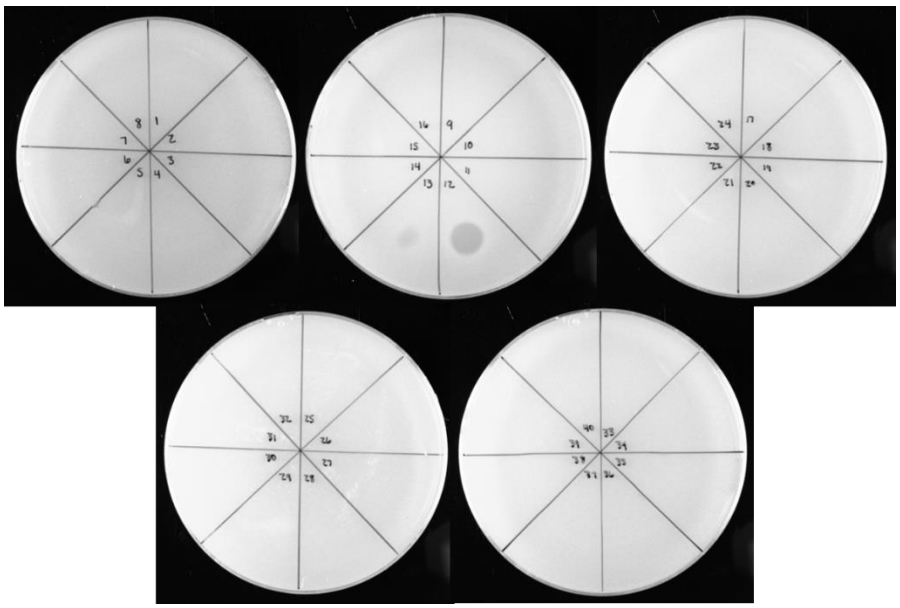

**Figure 2.8.** Bacteriocin activity from HPLC collected fractions.

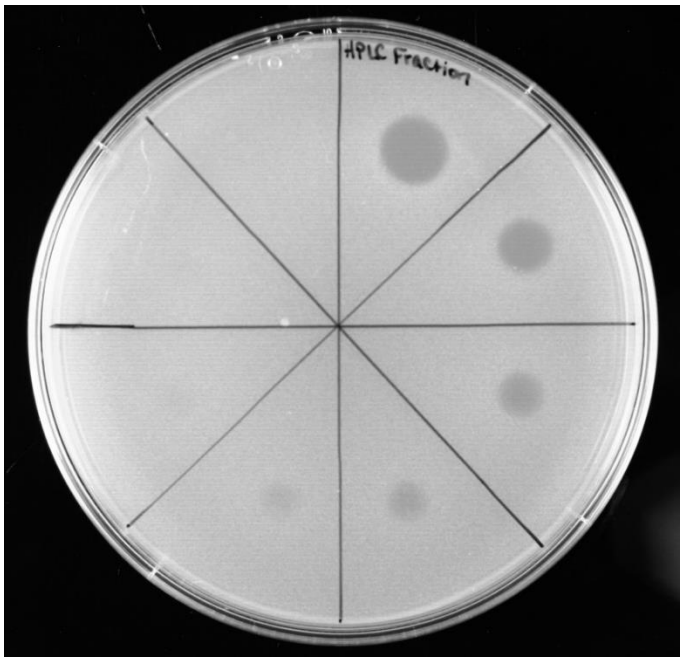


**Figure 2.9.** Bacteriocin activity from resuspended HPLC fraction #12 (22-24 minutes).

## CONCLUSION

Because lactic acid bacteria have GRAS status and can be used in foods, bacteriocins from these sources have become subjects of interest as antimicrobial measures against *L. monocytogenes* in food. In order to incorporate bacteriocins as antimicrobials in foods, each bacteriocin should be tested in each food for which it is intended. The main reason for this is that bacteriocins act differently in media than they do in food products, as the constituents within the food can affect the activity of the bacteriocins. These antimicrobial peptides are amphiphilic in nature and can bind to protein or fat and can degrade over storage periods (Budde et al., 2003; Hartmann et al., 2011). Bacteriocins within different foods could also react differently based on the amounts of various constituents within the foods.

The ability for *L. monocytogenes* to become resistant to bacteriocins has been noted (Gravesen et al., 2002; Kaur et al., 2011). The use of multiple mode-of-action (MOA) bacteriocin mixtures is a promising solution to this issue. Use of bacteriocins possessing different MOAs greatly decreases the likelihood of *Listeria* gaining bacteriocin resistance to the mixture. However, prior to implementation in foods, strains whose bacteriocins are to be used should be analyzed for virulence factors, as certain cultures can produce these.

For this study, the selection of spontaneously-resistant *L. monocytogenes* 39-2 allowed for the development of resistant isolates, which were then used to screen samples for the use of a novel MOA. This process yielded 6 isolates during the course of our studies. Isolates belonging to a genus that could potentially contain virulence factors were analyzed for hemolysin and gelatinase production. Four of the strains analyzed produced gelatinase, while only the control organism produced hemolysin; none of these were chosen for use in our applications in further studies.

The *Streptococcus* spp. 323 isolate produced a bacteriocin with considerable antagonistic activity against the most resistant *Listeria* strain, was grown in M17 medium without Tween and purified

by ammonium sulfate precipitation, C18 sep-pack cartridge extraction, and reversed-phase HPLC isolation.

# CHAPTER III

## SHELF-LIFE STUDIES EVALUATING THE EFFECTIVENSS OF MULTIPLE MODE-OF-ACTION BACTERIOCIN COCKTAIL IN COMPARISON WITH COMMERCIAL NATURAL ANTIMICROBIALS IN HOTDOGS


### ABSTRACT

Consumer preferences for natural, ingredients and the implication of *Listeria monocytogenes* in recent outbreaks originating from RTE products have indicated the need for effective, naturally-derived antimicrobials to ensure the safety of such products. Lactic acid bacteria (LAB) produce bacteriocins that display antagonistic effects against *L. monocytogenes*, which may present a solution for use in foods targeting the organism.

Our objectives were to analyze a multiple MOA bacteriocin cocktail and commercial antimicrobials through shelf-life studies challenged with *L. monocytogenes*.

Previously, a *L. monocytogenes* strain resistant to three different MOAs was made, which was then utilized to screen samples for a bacteriocin possessing an uncommon MOA, leading to the isolation of *Streptococcus* spp. strain 323. Along with this strain, three other LAB operating by two different MOA (*Lactobacillus curvatus* Beef 3, *Lactococcus lactis* FLS-1, and *Pediococcus acidilactici* Bac 3) were selected, and their spent culture supernatants combined as a bacteriocin cocktail.

The bacteriocin cocktail was compared to commercial antimicrobials NovaGard® (Dow) and Durafresh™ 2016 (Kerry) in shelf life studies to compare the antimicrobial treatments on hotdogs. Studies were conducted using high and low inoculums of a four-strain cocktail of *Listeria monocytogenes* (*L. monocytogenes* 39-2, *L. monocytogenes* V7-2, *L. monocytogenes* 383-2, *L. monocytogenes* CW2) on hotdogs free of other antimicrobial compounds, which were manufactured within the FAPC pilot plant. After inoculation, hotdogs were stored at 5°C and sampled periodically by plating on MOX agar for *L. monocytogenes* and MRSA pH 5.5 for LAB.

The addition of a bacteriocin cocktail to hot dog surfaces proved sufficient for maintaining *Listeria* levels below 3.0 log for 6 and 8 weeks in high and low inoculum studies, respectively. Application of a bacteriocin cocktail within the meat matrix during manufacture kept *Listeria* levels below 3.0 log CFU/mL after 16 weeks. Bacteriocins exhibited significantly better inhibition than NovaGard® at both concentrations applied and performed better than Durafresh™ 2016 at higher concentrations.

Given the data gathered in this study, multiple MOA bacteriocins for use as a hurdle technology could effectively reduce levels of *Listeria* in RTE products.

## INTRODUCTION

RTE meats are particularly vulnerable to contamination with *Listeria monocytogenes*, a particularly dangerous pathogen responsible for approximately 260 deaths each year (Centers for Disease Control and Prevention, 2017). These products are of concern due to their long shelf life and favorable environment for *Listeria* growth. The pathogen is particularly problematic due to its ubiquitous nature, its salt tolerance, and its ability to grow at low temperatures. Contamination typically occurs post-processing before the product is packaged. While the food industry has

addressed this issue by applying lactates and diacetates to products, such chemicals do not align with consumer preferences.

Bacteriocins have been proposed as a natural alternative to these antimicrobials. Because LAB are GRAS ingredients, direct addition of the cultures producing the bacteriocins or the addition of their fermented products to foods requires no approval (Deegan et al., 2006). Not only do these naturally-occurring antimicrobial peptides produced by LAB prevent growth of *L. monocytogenes*, but they also are ideal for use in food products because they are heat stable (Perez et al., 2014). In other words, the addition of these compounds to foods would not have minimal effects on the flavor of the product itself and could be applied even prior to cooking product. By using bacteriocins, an effective natural intervention would be possible for industry, greatly decreasing the likelihood of *Listeria* growth in the product, should it become contaminated during manufacture.

Bacteriocins have already been implemented in several commercial products, including Nisaplin® and ALTA 2341™. While these products have antilisterial effects, they each include only one type of bacteriocin. This could become a serious issue, as *L. monocytogenes* can gain spontaneous resistance to bacteriocins. Fortunately, bacteriocins work by different MOAs, so when *Listeria* gains resistance to one bacteriocin, it is still susceptible to bacteriocins employing a different MOA. This was demonstrated by Macwana and Muriana (2011), who were able to identify bacteriocins based on MOA by using bacteriocin-resistant *L. monocytogenes* strains as a screening process.

By combining bacteriocins with different MOAs, any potential *Listeria* that may come into contact with this blend would have additional obstacles to overcome, thereby adding to the protection of the food to which the bacteriocins are applied. Such bacteriocin cocktails were previously researched by Vijayakumar and Muriana (2017); however, the effectiveness of such

bacteriocin blends when compared to commercial natural antimicrobials has not been evaluated, which would be vital information if multiple MOA bacteriocin cocktails were to be implemented in industry.

The objectives of this research were to a) evaluate the effectiveness of a surface-inoculated multiple MOA bacteriocin cocktail against a 4-strain mixture of *L. monocytogenes* when compared to NovaGard® and Durafresh™ 2016 in a hotdog shelf-life study and b) evaluate the effectiveness of alternative applications of the bacteriocin cocktail against the 4-strain blend of *Listeria monocytogenes* on hotdogs.

Results of this work could help determine the feasibility of use of multiple MOA bacteriocin cocktails in the food industry and determine the best bacteriocin application method to achieve *Listeria monocytogenes* reduction in RTE meats.

## MATERIALS AND METHODS

### *Preparation of Cultures for Use in Studies*

Master cultures of bacteriocin-producing lactic acid bacteria (LAB) from the Muriana laboratory collection, which were used in previous studies, were stored at -80°C in milk-based freezing medium containing 11% non-fat dry milk, 1% glucose, and 0.2% yeast extract. Similarly, *Listeria* strains were stored in 11% non-fat dry milk, 1% glucose, and 0.2% yeast extract. Cultures used as working stocks were made by inoculating 9 mL of either MRS (LAB cultures) or TSB (*L. monocytogenes* cultures) broths with 100-µL of culture and growing overnight for 16 hours at 30°C. These were then streaked for isolation, and a single colony was selected and grown for 16 hours at 30°C before preparing cultures for freezing. Cultures were centrifuged at 8000 rpm with a SS-34 rotor in a Sorvall® RC 5C Plus centrifuge for 10 minutes, supernatant was discarded, and the remaining pellet was resuspended in milk-based freezing medium as previously described for storage at -80°C. From these cultures, bacteria needed for studies were propagated twice overnight for 16 hours before use.

### *Formation of Multiple Mode of Action Bacteriocin Cocktail*

The multiple mode of action (MOA) bacteriocin cocktail was chosen based on the different MOA each bacteriocin displayed, as well as absence of virulence factors in the producing organism and amount of bacteriocin activity produced. Cultures from different MOAs were selected based on these criteria, resulting in a bacteriocin cocktail consisting of cell free supernatant (CFS) from *Pediococcus acidilactici* Bac 3, *Lactobacillus curvatus* Beef 3, *Lactococcus lactis* FLS-1, and *Streptococcus* spp. 323 (Table 3.1).

**Table 3.1.** Cultures used in this study

| Bacterium | Strain | Source |
|---|---|---|
| **Bacteriocin Cocktail 1** | | |
| *Pediococcus acidilactici* | Bac 3 | Muriana Culture Collection |
| *Lactobacillus curvatus* | Beef 3 | Vijayakumar & Muriana, 2017 |
| *Lactococcus lactis* | FLS-1 | Vijayakumar & Muriana, 2017 |
| *Streptococcus lutetiensis* | 323 | This Study |
| **Bacteriocin Cocktail 2** | | |
| *Lactobacillus curvatus* | FS47 | Garver and Muriana, 1993 |
| *Enterococcus faecium* | FS56-1 | Garver and Muriana, 1993 |
| *Pediococcus acidilactici* | Bac 3 | Muriana Culture Collection |
| *Lactobacillus curvatus* | Beef 3 | Vijayakumar & Muriana, 2017 |
| *Lactococcus lactis* | FLS-1 | Vijayakumar & Muriana, 2017 |
| ***Listeria monocytogenes* Cocktail** | | |
| *Listeria monocytogenes* | 39-2 | Muriana Culture Collection |
| *Listeria monocytogenes* | V7-2 | Muriana Culture Collection |
| *Listeria monocytogenes* | 383-2 | Muriana Culture Collection |
| *Listeria monocytogenes* | CW2 | Muriana Culture Collection |

Alongside this bacteriocin cocktail (Bac #1), another bacteriocin cocktail (Bac #2) previously created within our laboratory, included 3 MOA and used in Vijayakumar and Muriana (2017), was chosen to test on hotdogs as well (Table 3.1). Strains of *L. monocytogenes* were also selected to be combined in a 4-strain cocktail as challenge organisms (Table 3.1) for application to the surface of hotdogs.

***Production of Hotdogs***

Hotdogs were produced in the meat processing pilot plant of the Robert M. Kerr Food and Agricultural Products Center. The performance of bacteriocin cocktails were compared to the effectiveness of two different commercial antimicrobials, which included NovaGard® (Dow),

applied at 0.25% of the total formulation, and Durafresh™ 2016 (Kerry), applied at 1% of the total formulation. Kerry also requested testing Durafresh alongisde a celery powder product, Accel™ 2000 (recommended application between 0.15 and 0.85%), which they are now using in place of traditional cure. Formulations for all batches can be shown below in Tables 3.2 through 3.6. Using fat percentage estimates obtained from a FOSS FoodScan™ after grinding beef trim, lean pork, and pork belly separately, meat was blended together to formulate an approximately 17% fat hotdog.

**Table 3.2.** Formulation for control hotdogs / bacteriocin-soaked casing hotdogs

| Ingredient | Formula % | Lbs | Grams |
|---|---|---|---|
| Beef Trim | 12.71% | 4.50 | 2041.16 |
| Lean Pork | 37.43% | 13.25 | 6010.09 |
| Pork Belly | 20.48% | 7.25 | 3288.54 |
| Water | 26.70% | 9.45 | 4286.44 |
| Seasoning | 2.47% | 0.88 | 396.89 |
| Cure (6.25% NO2) | 0.18% | 0.06 | 28.30 |
| Sodium Erythrobate | 0.03% | 0.01 | 4.54 |
| Total | 100% | 35.40 | 16055.96 |

**Table 3.3.** Formulation for hotdogs with bacteriocin addition in meat matrix

| Ingredient | Formula % | Lbs | Grams |
|---|---|---|---|
| Beef Trim | 12.72% | 6.43 | 2916.60 |
| Lean Pork | 37.39% | 18.90 | 8572.89 |
| Pork Belly | 20.50% | 10.36 | 4699.21 |
| Bacteriocin Mix | 26.71% | 13.50 | 6123.49 |
| Seasoning | 2.47% | 1.25 | 566.99 |
| Cure (6.25% NO2) | 0.18% | 0.09 | 40.51 |
| Sodium Erythrobate | 0.03% | 0.01 | 6.49 |
| Total | 100% | 50.54 | 22926.19 |

**Table 3.4.** Formulation for NovaGard hotdogs

| Ingredient | Formula % | Lbs | Grams |
|---|---|---|---|
| Beef Trim | 12.68% | 4.50 | 2041.17 |
| Lean Pork | 37.33% | 13.25 | 6010.09 |
| Pork Belly | 20.43% | 7.25 | 3288.54 |
| Water | 26.63% | 9.45 | 4286.49 |
| Seasoning | 2.47% | 0.88 | 396.89 |
| NovaGard | 0.25% | 0.09 | 40.38 |
| Cure (6.25% NO2) | 0.18% | 0.06 | 28.30 |
| Sodium Erythrobate | 0.03% | 0.01 | 4.51 |
| Total | 100% | 35.49 | 16096.37 |

**Table 3.5.** Formulation for Durafresh 2016 with chemical sodium nitrite hotdogs

| Ingredient | Formula % | Lbs | Grams |
|---|---|---|---|
| Beef Trim | 12.57% | 4.50 | 2038.87 |
| Lean Pork | 37.06% | 13.25 | 6010.09 |
| Pork Belly | 20.28% | 7.25 | 3288.53 |
| Water | 26.43% | 9.45 | 4286.48 |
| Seasoning | 2.45% | 0.88 | 396.89 |
| Durafresh | 1.00% | 0.36 | 162.74 |
| Cure (6.25% NO2) | 0.18% | 0.06 | 28.30 |
| Sodium Erythrobate | 0.03% | 0.01 | 4.51 |
| Total | 100% | 35.75 | 16216.43 |

**Table 3.6.** Formulation for Durafresh 2016 with Accel 2000 hotdogs

| Ingredient | Formula % | Lbs | Grams |
|---|---|---|---|
| Beef Trim | 12.53% | 4.50 | 2038.50 |
| Lean Pork | 36.94% | 13.25 | 6011.82 |
| Pork Belly | 20.21% | 7.25 | 3289.48 |
| Water | 26.35% | 9.45 | 4287.71 |
| Seasoning | 2.45% | 0.88 | 396.89 |
| Durafresh | 1.00% | 0.36 | 162.81 |
| Sodium Erythrobate | 0.03% | 0.01 | 4.51 |
| Accel | 0.50% | 0.18 | 80.97 |
| Total | 100% | 35.88 | 16272.69 |

Hotdog emulsions were prepared by combining ground lean meat, salt, water, and half of the seasonings, and mixing in a Seydelmann bowl chopper for 2 minutes. Then ground fatty meat was added, along with the remainder of the water and mixed until a temperature of 12.7°C (55°F) was achieved. No lactates or diacetates were applied to the mixture. The emulsion was then stuffed into 24/USA Viscofan cellulose casings, with the exception of a bacteriocin-soaked casings batch, which had 24/USA Viscofan cellulose casings soaked in bacteriocin cocktail #1 for 30 minutes prior to the stuffing step. Liquid smoke was then applied to the casings, ensuring the coating of every surface. Frankfurters were then placed in an Alkar RapidPak electric batch oven and the protocol listed below in Table 3.7 was applied. After hotdogs reached an internal temperature of 71.1°C (160°F), they were subjected to a cold shower, then cooled to a core temperature of 4.4°C (40°F). Product was vacuum-packaged and frozen until needed for shelf life studies.

**Table 3.7.** Hotdog cooking protocol within Alkar oven

| Dry Bulb Temperature (°F) | Wet Bulb Temperature (°F) | Time (minutes) |
|---|---|---|
| **130** | 93 | 20 |
| **160** | 128 | 20 |
| **175** | 145 | 20 |
| **190** | 165 | 5 |

***Preparation for Shelf Life Studies***

Bacteriocins were prepared by propagating each culture from frozen working culture twice for 16 hours at 30°C prior to centrifuging. After cells were eliminated, supernatants from each culture were mixed to obtain a crude bacteriocin cocktail. The cocktail was then pasteurized at 80°C for 15 minutes to remove any remaining cells, then stored at 4°C prior to use. A two-fold dilution titer assay, as previously described, was used to ensure activity of the mixed bacteriocin cocktail.

Four strains of *L. monocytogenes* (Table 3.1) were propagated twice for 16 hours at 30°C. Prior to application on hotdogs, the separate strains were mixed together in equal amounts and diluted to the desired inoculum concentration in order to achieve a 3 or 4 log CFU/mL when applied to the hotdogs, depending on the study being conducted.

Frozen hotdogs were thawed at 4°C overnight prior to use. A vat pasteurizer with temperature controls was used to pasteurize hotdogs at 80°C for 5 minutes. This was done remove any incidental background lactic acid bacteria that could potentially grow during refrigerated shelf-life storage and contribute to *Listeria* inhibition. Frankfurters were then allowed to cool on ice for one hour prior to inoculation.

Pasteurized, cooled hotdogs were aseptically transferred to 8 x 10-in. high barrier vacuum packages (Prime Source, Kansas City, MO; 3 mil high barrier nylon vacuum pouch, Item No. 75001942) with two frankfurters allotted per package. Each package had 300 µL of bacteriocin cocktail applied to the surface of the hotdogs, which were then hand-massaged to distribute bacteriocin. In the case of the control batch and commercial antimicrobial batches, frankfurters instead had 300-µL of DI water applied to the surface to keep moisture levels between all batches the same.

The *L. monocytogenes* 4-strain cocktail was then applied to all hotdog packages, in a 100-µL volume. The hotdogs were again hand-massaged to distribute the inoculum. Frankfurters were then vacuum-packaged and kept at 5°C until required for sampling.

***Sampling Procedure***

Test packages of frankfurters were sampled in triplicate at 0, 1, and 3 days, followed by weekly sampling until the 4-week period, then biweekly sampling periods for up to 20 weeks. For each sampling period, 3 random packages were withdrawn from the incubator and sanitized with alcohol before slicing a hole in one corner. Through this opening, 3-mL of 0.1% buffered peptone water (BPW) was added to the frankfurters. The hotdogs were then hand-massaged to promote dispersal of the *Listeria* into the liquid. The liquid was then extracted, treating this as the $10^0$ dilution, and then further 10-fold dilutions were made with 0.1% BPW. Dilutions were plated onto Modified Oxford Medium agar (MOX) to enumerate *L. monocytogenes*, as well as pH 5.5 DeMan, Rogosa, and Sharpe agar (MRSA) to enumerate potential LAB from the frankfurters.

## RESULTS AND DISCUSSION

### *Formation of Multiple Mode of Action Mixed Bacteriocin Cocktail*

Classification of bacteriocins by mode of action (MOA) and determination of the amount of activity each bacteriocin displayed against *L. monocytogenes* strains allowed for the formation of multiple MOA bacteriocin cocktail #1 for use in this study. Strains chosen for the multiple MOA bacteriocin cocktail represented 3 MOA and included *Streptococcus* spp. 323, *Lactococcus lactis* FLS-1, *Lactobacillus curvatus* Beef 3, and *Pediococcus acidilactici* Bac3. Cell-free supernatants (CFS) of these strains were combined and evaluated against each *Listeria monocytogenes* strain chosen for use in hotdogs using a serial two-fold dilution titer assay (Figure 3.1). From the titer assay, the inhibitory activity units (AU) against each *Listeria* strain were calculated (Table 3.9).

**Table 3.8.** Strains of each bacteriocin cocktail used in this study

| **Bacteriocin-Producing Strain Used** | **Mode of Action Used** | | | |
|---|---|---|---|---|
| | **#1** | **#2** | **#3** | **#4** |
| **Bacteriocin Cocktail #1 (This Study)** | | | | |
| *Pediococcus acidilactici* Bac 3 | ✔ | | | |
| *Lactobacillus curvatus* Beef 3 | ✔ | | | |
| *Lactococcus lactis* FLS-1 | | | ✔ | |
| *Streptococcus* spp. 323 | | | | ✔ |
| **Bacteriocin Cocktail #2 (Vijayakumar & Muriana, 2017)** | | | | |
| *Pediococcus acidilactici* Bac 3 | ✔ | | | |
| *Lactobacillus curvatus* Beef 3 | ✔ | | | |
| *Lactobacillus curvatus* FS47 | ✔ | | | |
| *Lactococcus lactis* FLS-1 | | | ✔ | |
| *Enterococcus faecium* FS56-1 | | ✔ | | |

**Table 3.9.** Activity of bacteriocin cocktail #1 against *L. monocytogenes* strains used in this study

| **Strain** | **Bacteriocin Cocktail #1 AU/mL** |
|---|---|
| *L. monocytogenes* V7-2 | 25,600 |
| *L. monocytogenes* 383-2 | 25,600 |
| *L. monocytogenes* 39-2 | 25,600 |
| *L. monocytogenes* CW2 | 12,800 |

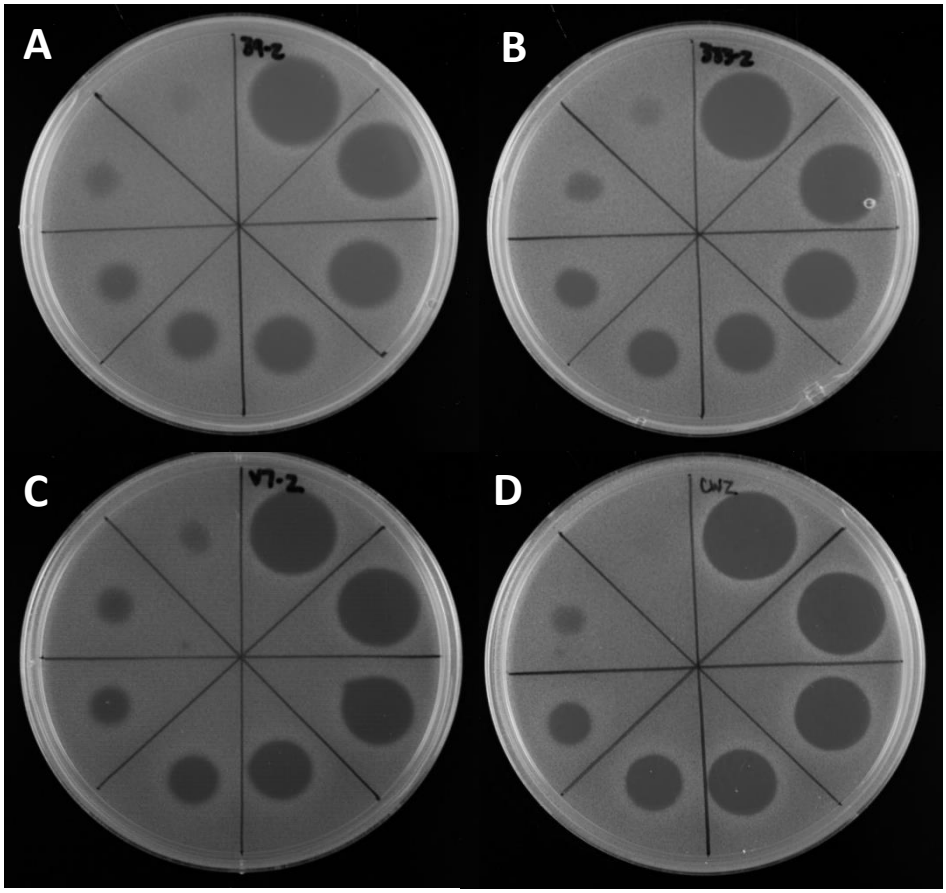


**Figure 3.1.** Two-fold dilution bacteriocin cocktail titer assay against strains of *Listeria monocytogenes* used in this study. Panel A, bacteriocin cocktail (*Pediococcus acidilactici* Bac3, *Lactobacillus curvatus* Beef 3, *Lactococcus lactis* FLS-1, and *Streptococcus* spp. 323) activity against *L. monocytogenes* 39-2; Panel B, bacteriocin cocktail activity against *L. monocytogenes* 383-2; Panel C, bacteriocin cocktail activity against *L. monocytogenes* V7-2; Panel D, activity of bacteriocin cocktail against *L. monocytogenes* CW2. This is the visual representation of bacteriocin activity units displayed in Table 3.9.

### *Production of Hotdogs*

Hotdogs were chosen for use in this study due to their high risk of contamination with *L. monocytogenes* (Center for Food Safety & Applied Nutrition et al., 2003). The susceptibility of product contamination with *Listeria* combined with their long shelf life made them an ideal candidate to test the application of bacteriocins. Post-processing contamination could be catastrophic for such foods due to the ability of the pathogen to grow at refrigerated temperatures. In order to evaluate the effectiveness of the multiple MOA bacteriocin cocktail, it also was essential to exclude lactates and diacetates from the hotdog formulation. While lactates and diacetates are commonly used in industry as preventive measures against *L. monocytogenes*, the incorporation of other antimicrobials would not demonstrate the true antimicrobial potential of the bacteriocin or commercial antimicrobial being tested.

### *Preparation for Shelf Life Studies*

After culturing bacteriocin-producing cultures, supernatants were prepared and combined. The bacteriocin cocktails (Bac#1 and Bac#2) were then evaluated against the *Listeria* strains to be used in the studies for each trial using a two-fold dilution titer assay. Inhibitory AU were noted for each study, and the results of each are shown below in Figure 3.2 and Table 3.10.

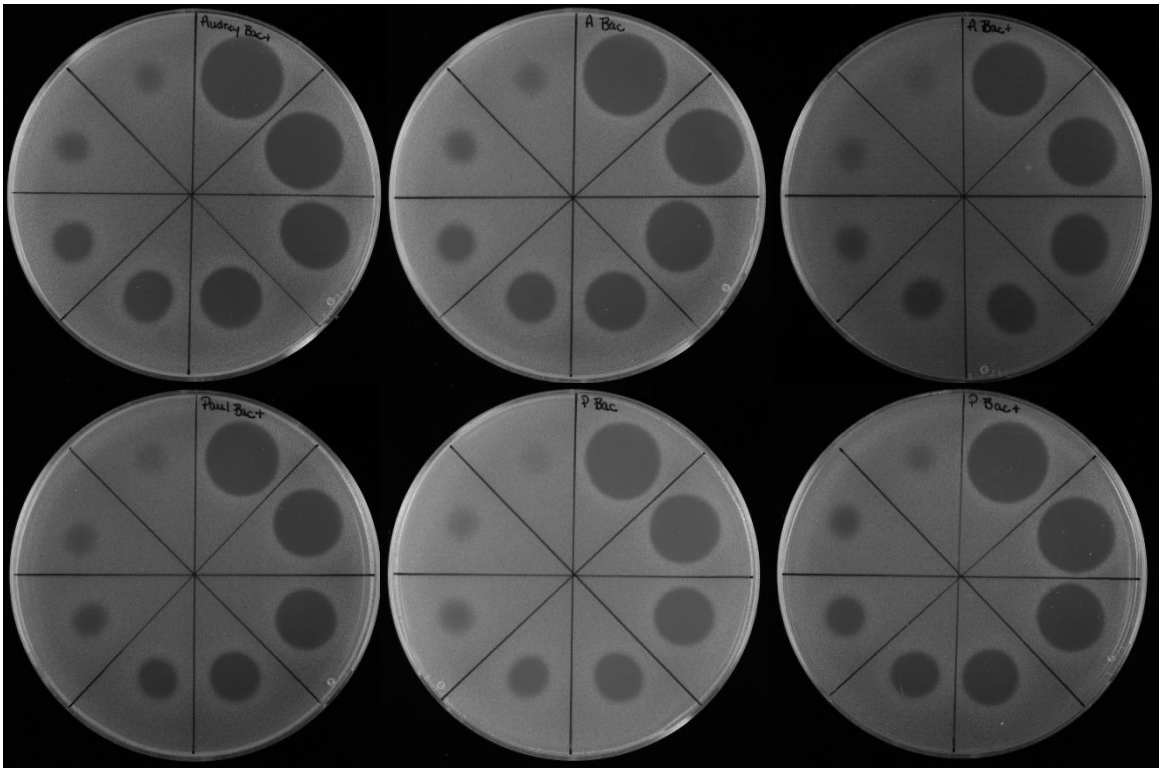


**Figure 3.2.** Activity of bacteriocin cocktails applied to each hotdog shelf life challenge study. Panel A, Bacteriocin Cocktail #1 (this study) activity against *Listeria monocytogenes* cocktail for high inoculation hotdog challenge study; Panel B, Bacteriocin Cocktail #1 (this study) activity against *L. monocytogenes* cocktail for low inoculation hotdog challenge study; Panel C, Bacteriocin Cocktail #1 (this study) activity against *L. monocytogenes* cocktail for alternative bacteriocin application and bacteriocin within meat matrix hotdog challenge study; Panel D, Bacteriocin Cocktail #2 (Vijayakumar & Muriana, 2017) activity against *L. monocytogenes* cocktail for high inoculation hotdog challenge study; Panel E, Bacteriocin Cocktail #2 (Vijayakumar & Muriana, 2017) activity against *L. monocytogenes* cocktail for low inoculation hotdog challenge study; Panel F, Bacteriocin Cocktail #2 (Vijayakumar & Muriana, 2017) activity against *L. monocytogenes* cocktail for alternative bacteriocin application and bacteriocin within meat matrix hotdog challenge study.

**Table 3.10.** Activity of bacteriocin cocktails applied in each shelf life study

| Study | Bacteriocin Cocktail #1 (This Study) | Bacteriocin Cocktail #2 (Vijayakumar & Muriana, 2017) |
|---|---|---|
| Study 1: Surface Application with High *Listeria monocytogenes* inoculation | 25,600 | 25,600 |
| Study 2: Surface Application with Low *Listeria monocytogenes* inoculation | 25,600 | 25,600 |
| Study 3: Bacteriocin-Soaked Casings and Bacteriocin Applied within Meat Matrix | 25,600 | 25,600 |

***Sampling Procedure and Analysis***

Previously, Vijayakumar and Muriana (2017) found the use of acidified MRS agar allowed the growth of lactic acid bacteria but inhibited the growth of *L. monocytogenes*, while MOX allowed the growth of *Listeria* but inhibited the growth of lactic acid bacteria. Prior to the hotdog trials in this study, strains were tested on these agars to confirm growth only occurred of desired organisms on each medium. After successfully confirming the validity of the test, acidified MRS agar was used to test for the presence of contaminating LAB in the studies conducted, while MOX was used to enumerate the inoculated *L. monocytogenes*. At each sampling period, 3 packages were sampled and plated in duplicate. The graphs depicting the data collected are shown below in Figures 3.3, 3.4, and 3.5. Statistical analysis was performed using SigmaPlot 13 One-Way Repeated Measures ANOVA, with significant differences among data being determined at $p < 0.05$.

***Shelf Life Study***

Three hotdog shelf-life challenge studies in were conducted using the 3 MOA bacteriocin cocktail formulated for this study (Bac #1), as well as the multiple MOA bacteriocin cocktail (Bac #2)

used in Vijayakumar and Muriana (2017). In addition, the effects of two commercial antilisterial products were evaluated, as well as a comparison between chemical sodium nitrite and a natural celery alternative (Accel 2000) with respect to their influence on *L. monocytogenes*. Figures below show results, along with discussion of the results of each study.

*Listeria monocytogenes* has been implicated in outbreaks with ready-to-eat (RTE) foods (Gombas et al., 2003), and many studies have found bacteriocins to be effective in meat models against *L. monocytogenes* (Chen et al., 2004; Du et al., 2017; Murray & Richard, 1997; Nieto-Lozano et al., 2010; Vijayakumar & Muriana, 2017). Therefore, evaluation of multiple MOA bacteriocin cocktails in hotdogs is pertinent research for the improvement of food safety.

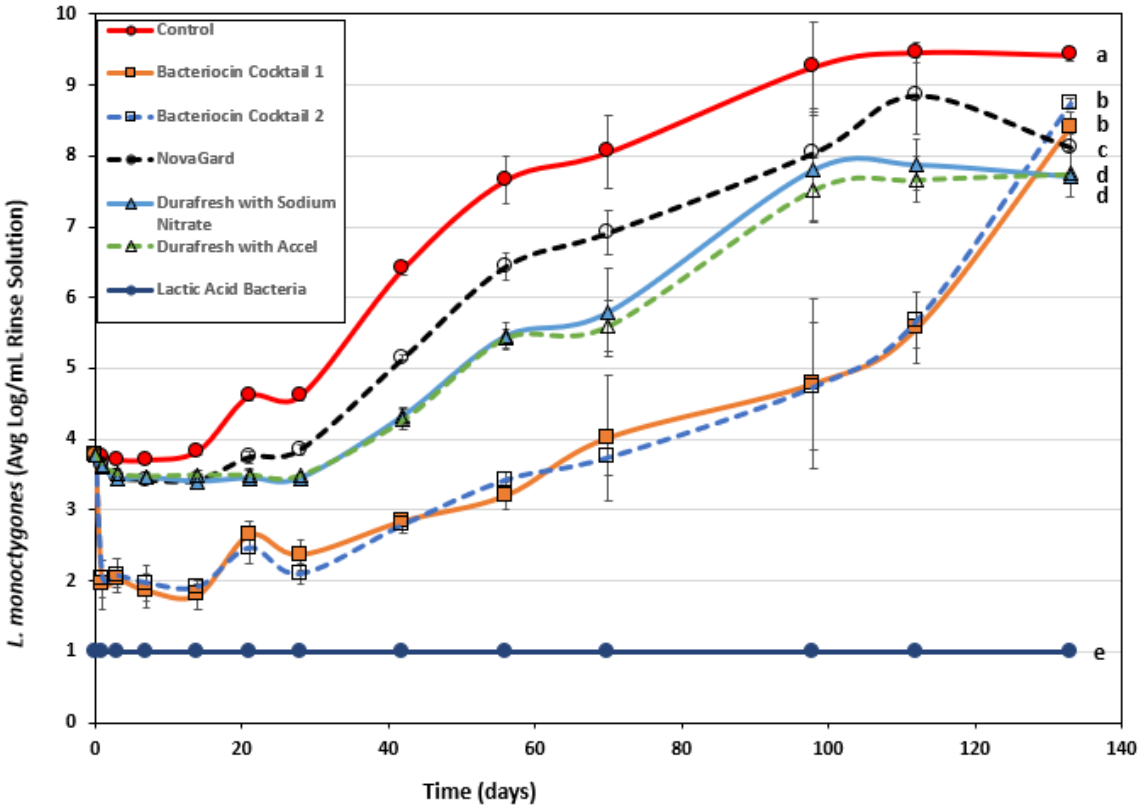


**Figure 3.3.** Hotdog challenge study with high inoculation of *Listeria monocytogenes* 4-strain cocktails. A 4-strain cocktail of *L. monocytogenes* was inoculated on hotdogs at 4 log CFU/mL by itself (control), with bacteriocin cocktail #1 (*Lactobacillus curvatus* Beef 3, *Pediococcus acidilactici* Bac3, *Lactococcus lactis* FLS-1, *Streptococcus* spp. 323), bacteriocin cocktail #2 (*Enterococcus faecalis* FS56-1, *Lb. curvatus* Beef 3, *P. acidilactici* Bac3, *Lc. lactis* FLS-1, and *Lb. curvatus* FS47), NovaGard, Durafresh 2016 with chemical sodium nitrite, or Durafresh 2016 with Accel 2000 (natural nitrite). All sample treatments were performed in triplicate replication; data points represent the mean and error bars represent the standard deviation from the mean. Treatment with different lowercase letters are significantly different (repeated measures ANOVA, $p < 0.05$).

***Study 1: High Inoculation with 4-Strain Listeria monocytogenes Cocktail***

The first shelf-life challenge study examined the effect of a high inoculum (4 log CFU/mL) of a 4-strain cocktail of *L. monocytogenes* on hotdogs with different antimicrobial treatments and a control, resulting in 6 different batches. These included NovaGard (Danisco, added at 0.25%), Durafresh 2016 (Kerry, added at 1.0%) using sodium nitrite (156 ppm) as the nitrite source, Durafresh 2016 (Kerry, added at 1.0%) using Accel 2000 as the nitrite source (Kerry, added at 100ppm), and two different bacteriocin cocktails, bacteriocin cocktail #1 (CFS from *Lactobacillus curvatus* Beef 3, *Pediococcus acidilactici* Bac 3, *Lactococcus lactis* FLS-1, and *Streptococcus* spp. 323) and bacteriocin cocktail #2 (CFS from *Lactobacillus curvatus* FS47, *Enterococcus faecium* FS56-1, *Lactobacillus curvatus* Beef 3, *Pediococcus acidilactici* Bac 3, and *Lactococcus lactis* FLS-1).

Statistical analysis indicated a significant difference ($p < 0.05$) between the control and all other treatments over the course of the study. Bacteriocin treatments did not significantly differ from each other, and similarly, the Durafresh 2016 batches comparing the use of chemical sodium nitrite and Accel 2000 (natural nitrite) had no significant difference from each other. A significant difference ($p < 0.001$) was observed between the bacteriocin treatments and both Durafresh 2016 batches. NovaGard had a significant difference when compared to both Durafresh 2016 batches and both bacteriocin batches ($p < 0.001$). Growth of LAB did not occur at any point during the study, indicating the effectiveness of the pasteurization step (Figure 3.3).

A loss of bacteriocin activity during storage or when applied to a food has been well-documented and is not unusual (Aasen et al., 2003; Kouakou et al., 2009; Woraprayote et al., 2016). Bacteriocins have the ability to bind to components within a food, such as proteins and fat. Other factors can also contribute to loss of bacteriocin activity, including proteolytic activity and oxidation (Aasen et al., 2003; Kouakou et al., 2009). To illustrate this, a study conducted by

Aasen et al. (2003), determined bacteriocin activity of sakacin P before and after incubation for 4 weeks in salmon and chicken. Results of the study found an approximate loss of 80% of the sakacin P activity and an average loss of 2-µg/g in salmon and 3-4 µg/g in chicken models. The study also observed a higher recovery rate of bacteriocin activity at more acidic conditions tested, though degradation of bacteriocin activity still occurred with these conditions. Given this information, it is a logical conclusion that the increase in *L. monocytogenes* observed in this study could be a result of bacteriocin degradation or binding to constituents in the hotdogs.

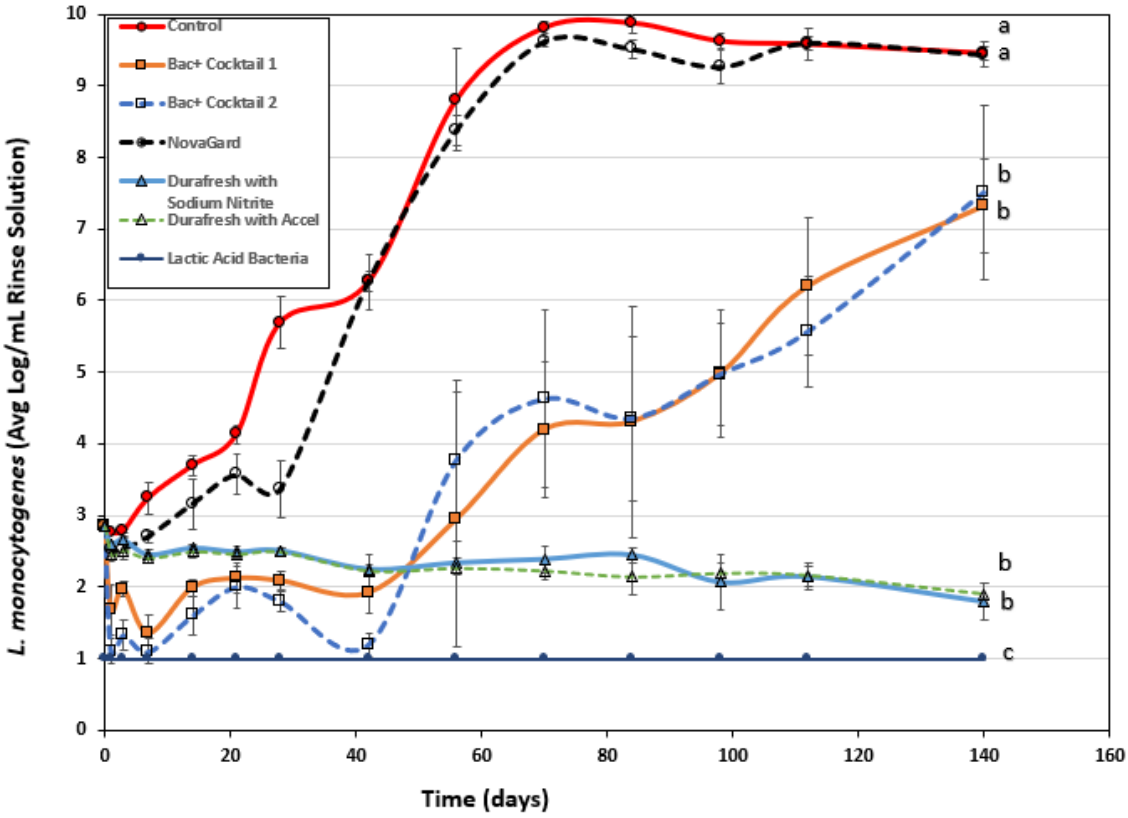


**Figure 3.4.** Hotdog challenge study with low inoculation of *Listeria monocytogenes* 4-strain cocktail. A 4-strain cocktail of *L. monocytogenes* was inoculated on hotdogs at 3 log CFU/mL by itself (control), with bacteriocin cocktail 1 (*Lactobacillus curvatus* Beef 3, *Pediococcus acidilactici* Bac3, *Lactococcus lactis* FLS-1, *Streptococcus* spp. 323), bacteriocin cocktail 2 (*Enterococcus faecalis* FS56-1, *Lb. curvatus* Beef 3, *P. acidilactici* Bac3, *Lc. lactis* FLS-1, and *Lb. curvatus* FS47), NovaGard, Durafresh 2016 with chemical sodium nitrite, or Durafresh 2016 with Accel 2000 (natural nitrite). All sample treatments were performed in triplicate replication; data points represent the mean and error bars represent the standard deviation from the mean. Treatment with different lowercase letters are significantly different (repeated measures ANOVA, $p < 0.05$).

### *Study 2: Low Inoculation with 4-Strain Listeria monocytogenes Cocktail*

Study 2 observed the effect of a low inoculum (3 log CFU/mL) of the 4-strain *L. monocytogenes* cocktail against the same antimicrobials used Study 1. For this, statistical analysis using One-Way Repeated Measures ANOVA indicated both bacteriocin batches showed a significant reduction from the control batch throughout the study ($p < 0.001$). Similarly, both Durafresh 2016 batches showed a significant reduction from the control throughout the study. Durafresh and bacteriocin treatments performed significantly better than NovaGard $p < 0.001$), showing lower levels of *L. monocytogenes.* The hotdogs treated with NovaGard did not show a statistically significant difference ($p = 0.866$) relative to the control but did statistically differ from the remaining hotdog batches ($p < 0.001$). Batches with bacteriocin applications did not differ significantly from each other ($p = 0.958$), while Durafresh 2016 batches comparing use of sodium nitrite to Accel 2000 showed no statistical difference from each other ($p =0 .924$). Growth of LAB was not observed during the study on acidified MRS agar, which proved the effectiveness of the pasteurization step to remove background bacteria (Figure 3.4).

### *Accounting for Differences Between Studies 1 and 2*

Differing results between hotdog batches in study 1 and study 2 were sources of concern. After proximate analysis of batches were performed by the Food and Agricultural Products Center Chemical Analysis laboratory (Table 3.11), results indicated fat percentages differed from each other by about 5 percent and protein differed by about 2 percent. This issue could have been avoided by formulating hotdogs for all studies at the same time with the same meat sources. This was not as much of a concern in study 3, as the proximate analysis for these hotdogs differed

from those in study 1 in the proximate analysis by only about 1% for both fat and protein amounts.

**Table 3.11.** Proximate analysis of hotdogs from each study

| Study | % Moisture | % Ash | % Fat | % Protein | % Carbohydrates |
|---|---|---|---|---|---|
| Study 1 Hotdogs | 63.61 | 2.84 | 17.81 | 14.22 | 1.52 |
| Study 2 Hotdogs | 67.33 | 2.78 | 12.11 | 16.12 | 1.66 |
| Study 3 Hotdogs | 62.20 | 2.25 | 18.30 | 15.24 | 2.01 |

***Similarities Between Use of Sodium Nitrite and Accel 2000***

The similarities between the Durafresh 2016 batch with sodium nitrite and the Durafresh 2016 with Accel 2000 were expected. Sodium nitrite and the celery powder from which the Accel 2000 containing nitrite is made are intended to inhibit the germination of *Clostridium* spp., rather than *L. monocytogenes*. When compared to the chemical sodium nitrite, the Accel 2000 showed no significant difference in the amount of *L. monocytogenes* in study 1 or study 2 ($p=0.926$, $p=0.924$ respectively). Kouakou et al. (2009) found a slight inhibition of *L. monocytogenes* by nitrites when used in a pork meat model totaling between a 1 and 2 log difference from the control batch after a 6-week incubation period. However, the research conducted in this study did not evaluate any hotdogs made without nitrites in order to evaluate the overall effect of nitrites on the level of *L. monocytogenes*. Therefore for the purposes of this study, no conclusions regarding inhibition of *L. monocytogenes* can be made.

***Concentration of Bacteriocin***

After seeing the late rise of *L. monocytogenes* in bacteriocin surface treatments, it was hypothesized that a higher concentration or a lower level of *L. monocytogenes* inoculum would result in better inhibition of the pathogen. The high concentration of bacteriocin also would be more comparable to the amount of NovaGard or Durafresh 2016 used in previous challenge studies, as these are freeze-dried products, while the bacteriocin present in bacteriocin cocktails are much more dilute. This led to the formulation of the hotdogs with bacteriocin replacing water within the hotdog meat emulsion. Hartmann et al. (2011), and Schillinger et al. (1996) discussed the issue of decreased bacteriocin activity in foods when compared to applications in media. To alleviate this issue, an increase in bacteriocin concentrations within foods would be a simple solution. Based on the results seen, activity of bacteriocin depends on the concentration of the antimicrobial within the matrix it is applied. This can be observed by the difference in bacteriocin batches in studies 2 and 3 (Figures 3.4 and 3.5, respectively), where the differences in the amount of bacteriocin applied to hotdogs resulted in large differences in the amount of *L. monocytogenes* inhibited. For instance, comparing Week 12 of studies 2 and 3, an approximately 3 log CFU/mL difference can be observed between the bacteriocin cocktail #2 surface application from study 2 and bacteriocin cocktail #2 added within the meat matrix in study 3 (Figures 3.4 and 3.5). While the high concentration of bacteriocin inhibits the growth of *L. monocytogenes* quite well, the cost of incorporating such a high amount may not be feasible for use in industry. Since contamination largely occurs post-processing, surface applications, such as those used in studies 1 and 2 of this paper have been a subject of focus, but by soaking casings in bacteriocin, the antimicrobial peptides would be in contact with the surface of the product prior to the peeling step, where most contamination occurs. Similarly, use of freeze-dried bacteriocin incorporated with packaging has also been suggested as a potential solution to prevent surface contamination (Quintavalla & Vicini, 2002).

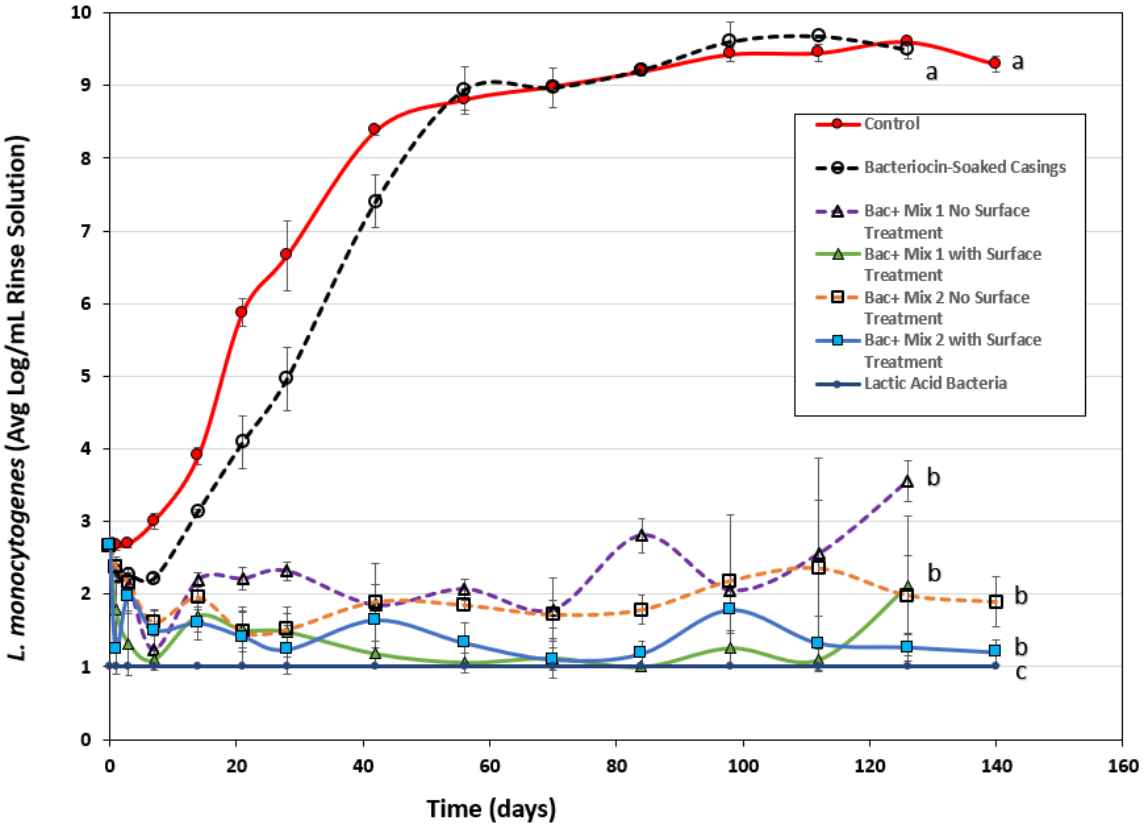


**Figure 3.5.** Hotdog challenge study with bacteriocin-soaked casings and meat matrix bacteriocin additions at low inoculation levels of *Listeria monocytogenes* 4-strain cocktail. A 4-strain cocktail of *L. monocytogenes* was inoculated on hotdogs at 3 log CFU/mL by itself (control), with bacteriocin cocktail #1 (*Lactobacillus curvatus* Beef 3, *Pediococcus acidilactici* Bac3, *Lactococcus lactis* FLS-1, *Streptococcus* spp. 323) within the meat matrix, bacteriocin cocktail #1 within the meat matrix with an additional surface application of the bacteriocin, bacteriocin cocktail #2 (*Enterococcus faecalis* FS56-1, *Lb. curvatus* Beef 3, *P. acidilactici* Bac3, *Lc. lactis* FLS-1, and *Lb. curvatus* FS47) within the meat matrix, bacteriocin cocktail #2 within the meat matrix with an additional bacteriocin surface application, and casings soaked in bacteriocin cocktail #1. All sample treatments were performed in triplicate replication; data points represent the mean and error bars represent the standard deviation from the mean. Treatment with different lowercase letters are significantly different (repeated measures ANOVA, $p < 0.05$).

***Study 3: Bacteriocin-Soaked Casings and Bacteriocins Applied within the Meat Matrix***

This study analyzed the effect of a low inoculum (3 log CFU/mL) of the 4-strain *L. monocytogenes* cocktail against hotdogs whose casings had soaked for 30 minutes in bacteriocin cocktail #1, as well as batches of hotdogs where the water component had been replaced with bacteriocin cocktail #1 or bacteriocin cocktail #2. The frankfurters testing the effect of the addition of bacteriocin to the meat matrix were split into two treatments; hotdogs with an additional 300 µL surface treatment of the respective bacteriocin cocktail, and hotdogs without the additional surface treatment.

Repeated Measures One-Way ANOVA indicated a significant difference between the control and all treatments where bacteriocin was added within the meat matrix (Figure 3.5). Batches that had bacteriocin applied within the meat matrix all had p-values less than 0.001 when compared to the control, indicating a significant difference in treatment. At Week 16 these batches all showed at least 6 log CFU/mL less *L. monocytogenes* from the control. All batches that had bacteriocin added within the meat matrix, regardless of the application of an additional surface treatment, had no significant difference from each other ($p < 0.001$). The batch with the bacteriocin-soaked casings showed no significant difference from the control batch, which gave a p-value of 0.984. Background bacteria were absent, as no LAB counts were observed on acidified MRS agar (Figure 3.5).

Use of non-neutralized bacteriocins within the meat matrix can cause the pH level to interfere with the meat matrix functionality and lead to product failure. This was observed during the addition of bacteriocin in place of water within the meat matrix for study 3. It is worth noting that pH levels between the batches with the bacteriocin cocktails were only separated by 0.13, but only the meat within the batch with the lowest pH level did not remain intact and fell apart after

the cooking process (Table 3.12). After observing these results, this batch was discarded. Upon increasing the pH of the bacteriocin supernatant to 6.0 prior to incorporation in the meat, no meat binding issues were observed with the final product and effective inhibition of the *L. monocytogenes* in the shelf-life study was still observed.

**Table 3.12.** Effect of non-neutralized cell-free supernatant containing bacteriocin on hotdog batter pH

| Batch | pH | Product Failure (Yes/No) |
|---|---|---|
| Control | 6.03 | No |
| Bacteriocin Mixture 1 (This Study) | 5.53 | Yes |
| Bacteriocin Mixture 2 | 5.66 | No |

The pH within a given product is important regarding the activity of a bacteriocin. Aasen et al. (2003) observed more bacteriocin activity recovery at lower pH levels, while Murray and Richard (1997) observed a better bactericidal effect at slightly acidic pH levels and higher growth rates of remaining live *L. monocytogenes*. Rodríguez et al. (2002) reported highest stability of pediocin PA-1 at pH levels between 4 and 6, but activity was lost during storage at pH levels above 7. Similarly, it has been reported that nisin loses activity above pH 7 (Deegan et al., 2006).

## CONCLUSION

The shelf-life trials conducted within this study show that a mixture of bacteriocin fermentates with different modes of action (MOA) could be applied as an effective antimicrobial ingredient for the inhibition of *L. monocytogenes* given a high enough concentration of the bacteriocin is present. After analysis with One-Way Repeated Measures ANOVA ($p < 0.05$), initial studies showed the surface application of 300 µL of a 3 MOA bacteriocin cocktail to hotdogs inhibited *L. monocytogenes* growth significantly when compared to control hotdogs ($p < 0.001$) at both high and low inoculum applications (Figure 3.3 and 3.4). Similarly, hotdogs formulated with the addition of Durafresh 2016 (Kerry) showed significant inhibition of *L. monocytogenes* ($p < 0.001$) in comparison with control hotdogs in the same studies, while NovaGard (Danisco) may have only had a statistically significant decrease from the control ($p=0.018$) at a 4 log CFU/mL inoculum level of *Listeria monocytogenes* (Figure 3.3) but the difference had little practical significance.

Treatments in studies 1 and 2 comparing the effect of sodium nitrite to Accel 2000 (Kerry), a celery powder used as a natural alternative to sodium nitrite, showed no significant additional inhibition from use of Accel 2000 powder on the *Listeria monocytogenes* 4-strain cocktail.

Replacement of water with mixed MOA bacteriocin cocktails within the meat matrix during formulation showed a large reduction (at least 6 log CFU/mL for all treatments) in comparison to control hotdogs, with all batches showing a significant difference from the control. Batches that had an additional 300 µL bacteriocin surface treatment showed the lowest levels of *L. monocytogenes* throughout the shelf life study. Bacteriocin-soaked hotdog casings inhibited *Listeria* growth for a short time but did not display a significant difference when compared to the control for the duration of the study.

Bacteriocin treatments were shown to be effective against a 4-strain *Listeria monocytogenes* cocktail and inhibited growth throughout the shelf life of the product. Given these results, use of bacteriocins could be applied in industry as a cheap, natural antimicrobial intervention to improve food safety. In future research, the application of freeze-dried bacteriocin preparations to hotdog casings should be examined. Freeze-dried bacteriocins could be easily applied as a powder, and a high concentration of soluble bacteriocin would be applied to each hotdog in this way. Such measures would address the issue of surface contamination with *L. monocytogenes* post-processing. In addition to this, evaluations of the amount of bacteriocin recovery after application to products would improve upon knowledge of how the bacteriocins react during shelf life when in contact with its intended food constituent, which would also improve upon research performed in this study.

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