ISOLATION AND PHYLOGENETIC ANALYSIS OF BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA FROM RETAIL FOODS

By

CHRISTOPHER DAVID HENNING

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Thesis Approved:

Peter M. Muriana

Thesis Adviser

Babu Fathepure

Divya Jaroni

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Abstract: Lactic acid bacteria (LAB) are known producers of small, anti-microbial peptides termed bacteriocins. The first study looked at the presence of bacteriocin producing (Bac+) LAB on unprocessed retail foods. A total of 170 food samples from 108 different food products yielded 43 isolates with antimicrobial activity against *Listeria monocytogenes*. Isolated Bac+ LAB included *Lactococcus lactis, Lactobacillus curvatus, Carnobacterium maltaromaticum, Enterococcus faecium,* and *Leuconostoc mesenteroides,* including two Gram-negative bacteria, *Serratia plymuthica*, and *Serratia ficaria*. A wide variety of food products contain Bac+ bacteria, but the majority were isolated from fresh vegetables. These data propose that Bac+ LAB are widely dispersed as part of the natural flora of unprocessed foods.

A second study examines the bacteriocin structural gene sequences of *Enterococcus* strains isolated from food as well as additional strains from animal sources. This second study utilizes a PCR primer array containing 16 primer pairs to detect bacteriocin structural genes in 22 Enterococcus spp isolates. Each isolate contained at least one of the screened structural gene with 15 of the 22 containing at least two. Enterocin A (entA), enterocins mr10A and mr10B (mr10AB), and bacteriocin T8 (bacA) were the most commonly found structural genes in order of decreasing prevalence. Our results display a high degree of bacteriocinogenic potential among enterococci which promise a part in biopreservation of food.

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CHAPTER I

REVIEW OF LITERATURE

Introduction

In 2001, the United States Centers for Disease Control and Prevention (CDC) estimated that cases of foodborne illness had reached numbers totalling 76 million (Cleveland et al., 2001). Of those cases, roughly 5000 estimated deaths had occurred (Cleveland et al., 2001). One category of foodborne illness, listeriosis, is a severe bacterial infection caused by the consumption of food contaminated with the bacterium *Listeria monocytogenes*. Listeriosis is a high-risk illness among immunocompromised individuals such as the elderly, pregnant women, and children although it still presents a serious risk for other individuals with mortality rates ranging from 23 to 44 percent (Williams and Chanos, 2012). Other serious foodborne pathogens include *Campylobacter jejuni, Clostridium perfringens, Escherichia coli* O157:H7, *Salmonella* spp., *Staphylococcus aureus,* and *Toxoplasma gondii (Cleveland et al., 2001).* The total estimated yearly cost for treatment of all foodborne illness is between 6.5 and 34.9 billion dollars according to the CDC (Cleveland et al., 2001).

While chemical treatments can adequately control the growth of some foodborne

pathogens, there is an increasing demand among consumers for food that does not contain what is deemed by them as harsh chemicals and which utilizes more mild and natural treatment (Wieckowicz et al., 2011). Therefore, the food industry and their consumers need new technology to combat contamination of food by foodborne pathogens while appeasing consumers' desire for more natural products (Wieckowicz et al., 2011). Many papers have been written regarding the replacement of chemical methods of preservation in food including the use of ribosomally synthesized antimicrobial peptides (i.e. bacteriocins) in food (Casaus et al., 1997; Macwana and Muriana, 2012). The world food supply relies strongly on the availability of fermented foods in the market that rely on the presence of lactic acid, but more recent studies have examined additional enhancement by antimicrobial peptides also produced by starter cultures (Nes et al., 2006; Nes and Johnsborg, 2004). It is thought that bacteriocins have likely been consumed for centuries as fermented foods have a long history of consumption around the world (Cleveland et al., 2001). Many bacteriocins found in food today are likely added unintentionally as a result of growth of the natural flora in food but also as a side product of added probiotics (Garver and Muriana, 1993; Nes et al., 2006). Not only do bacteriocins display antagonistic activity towards foodborne pathogens but also show activity against spoilage organisms (Knoll et al., 2008; Macwana and Muriana, 2012). Bacteriocins have varying inhibitory spectra, but provide a competitive and protective role against others species that are usually closely related (de Jong et al., 2006; Knoll et al., 2008). Bacteriocins are naturally produced by bacteria and found in all major groups of bacteria and archaea (Gillor et al., 2008; Rince et al., 1997). Bacteriocins were first studied and discovered among the Gram-negative *Escherichia coli*. These colicins, as bacteriocins produced by

E. coli were named, have a large range of activity against other bacteria, especially those that were closely related (Cleveland et al., 2001). Lactic acid bacteria (LAB) are particularly of interest among the bacteria which produce bacteriocins. LAB are considered "generally recognized as safe" (GRAS) by the United States Food and Drug Administration (FDA). While bacteriocins produced by the LAB are not automatically granted their GRAS status, the bacteria found in the food are still able to produce these compounds. LAB such as *Lactobacillus* spp. are also well documented to have a probiotic effect when ingested from food (Casaus et al., 1997). In addition to bacteriocins, LAB are also seen to produce other inhibitory compounds such as reuterin and terutericyclin which inhibit a much broader range of organisms including fungi (Nes and Johnsborg, 2004). Typically, bacteriocins produced by LAB will only be effective against Gram-positive bacteria because Gram-negative bacteria are protected against most of their activity by the outer membrane (Liu et al., 2011). Even the bacteriocins which are able to inhibit Gram-negative bacteria do not have a broad range of activity (Liu et al., 2011). Another significant inhibitor to pathogenic and spoilage bacteria is the production of lactic acid by LAB (Casaus et al., 1997). Besides being inhibitors of foodborne pathogens, bacteriocins can be used in tandem with other antimicrobial treatments in what is known as the hurdle effect in order to increase preservation of food (Cleveland et al., 2001). For example, the antimicrobial effect of nisin is enhanced when mixed with a metal chelating molecule like EDTA or a pulsed electric field which can also greatly increase its effectiveness against Gram-negative bacteria due to temporary pores being formed in their outer membrane (Cleveland et al., 2001). Full suppression of

target organisms with bacteriocins can be rarely achieved due to inability to be fully distributed within a food matrix (Casaus et al., 1997).

Nisin and its Use

One example of a well-studied bacteriocin is nisin (Casaus et al., 1997), which displays antagonistic activity against a few major Gram-positive foodborne pathogens: *Listeria monocytogenes, Clostridium botulinum, Staphylococcus aureus,* and *Bacillus cereus* (Abee et al., 1995). More recent studies have shown that Nisin may not be as effective against *Listeria monocytogenes* as other bacteriocins produced by LAB (Himeno et al., 2012). Cleveland et. al (2001), showed that 35 out of 40 tested isolates of *Lactococcus lactis* produce nisin. Nisin is used as a direct food additive in 40 countries and has been used for an excess of 50 years (Casaus et al., 1997). Nisin has been sold commercially as Nisaplin® since 1953 (Williams and Chanos, 2012) and is used as a food preservative in more than 50 countries (Himeno et al., 2012). Notably, pediocin PA-1, produced by LAB *Pediococcus acidilactici* PAC 1.0, is also sold for commercial use although its inhibitory spectrum is not as broad as nisin (Casaus et al., 1997). Currently, nisin is the only bacteriocin allowed by the FDA to be used as a food additive. Other bacteriocins produced by LAB can be used as crude mixtures in "cultured whey" and "cultured milk" ingredients. Nisin has a history of being used in meat, where its producer *L. lactis* is naturally found (Cleveland et al., 2001). Unfortunately, Nisin has been shown to be potentially unstable pH values greater than 5, making it not optimal for use in meats which have higher pH values (Casaus et al., 1997). The use of nisin in meat can reduce

the amount of required nitrite. Nitrites are known to prevent sporulation of the anaerobic spoilage and toxigenic bacteria *Clostridium* spp. (Cleveland et al., 2001). However, nisin cannot replace nitrites entirely because its universal distribution within meat cannot be practically achieved, and nisin shows a significantly higher solubility in low pH (Cleveland et al., 2001). Cloning of nisin into a *Lactococcus lactis* starter culture for Gouda cheese has been demonstrated. Sporulation of *Clostridium tyrobutyricum* was completely inhibited when a bacteriocin-producing starter culture was used (Abee et al., 1995). In addition, nisin can prevent the germination of *Clostridium botulinum* spores. Cleveland et al. (2001) speaks briefly about the concern for use of nisin in food and the increasing generation of cross-resistance of foodborne pathogens to vancomycin and several other antibiotics. This concern is unfounded as bacteria that have been repeatedly exposed to nisin retain their resistances to vancomycin, ampicillin, and chloramphenicol (Cleveland et al., 2001). It has also been shown that penicillin-resistant *Staphylococcus aureus* is 50 times more sensitive to nisin; however, resistance to nisin in *Listeria monocytogenes* is more dependent on lipid composition rather than an expressed immunity gene (Cleveland et al., 2001).

Bacteriocin Structure

Many bacteriocins have been studied and categorized based on their amino acid sequence, composition, and inhibitory spectra. Some common features of bacteriocins include the presence of cationic charges, amphiphilic conformations, and interactions with target membranes (Knoll et al., 2008). Bacteriocins also follow a common amino

acid makeup including low cysteine content with most molecules having one pair or less, low proline content with 74 percent of bacteriocins including one or fewer proline residue, net positive charge ranging from +1 to +12 and less than 12 percent having a negative charge, and a high hydrophobic residue content (Hammami et al., 2007). Bacteriocins start as larger molecules but are post-translationally cleaved of 18-24 amino acids which most likely constitutes a signal or recognition sequence for the export or relocation of the proteins (Allison et al., 1994). For example, enterocin B, a bacteriocin synthesized by *Enterococcus faecium*, is originally 71 amino acids but becomes cleaved down to 53 amino acids before it is actived by an ATP-binding cassette (Casaus et al., 1997; Cintas et al., 1998). In addition, enterocin B displays a double glycine motif just before the cleavage site which is a common feature among bacteriocins (Casaus et al., 1997). This consensus double glycine is a common N-terminal recognition sequence in class 2 bacteriocins, discussed in the next section, which is used to help secrete the newly synthesized peptide (Cintas et al., 1998). Other bacteriocins are secreted by a more general secretory pathway possibly involving a positively charged N-terminus and a hydrophobic core (Cintas et al., 1998).

Bacteriocin Classes

Bacteriocins have been divided into various categories or classes based on common features including conserved biochemical and structural properties, although some features cause them to be classified incongruently across researchers. De Jong et al. (2006) describes five factors used to classify bacterions which include homology to other

classified bacteriocins, presence of domains seen in members of the same class, bacteriocin size, and isoelectric point. In contrast, Zouhir et al. (2010) describe the classification of bacteriocins as being based on activity, method of excretion, amino acid sequence, and mode of action. The current method(s) of classifying bacteriocins is inconsistent and current confirmations of novel bacteriocins is reliant uponamino acid and gene sequences (Chang et al., 2013). This is compounded by different researchers discovering the same bacteriocin at different times and giving them different names. Of 107 individual bacteriocins, 40 fell in one class and subclass while 20 fell into more than one class and subclass at the same time (Zouhir et al., 2010).

Class I bacteriocins, known as lantibiotics, are unique because of the presence of unusual amino acids in their structures including lanthionine, methyl-lanthionine, dehydrobutyrine, and dehydroalanine (Cleveland et al., 2001; Wieckowicz et al., 2011). These bacteriocins are translated into precursor molecules which under extensive posttranslational modifications to create a final product (Chang et al., 2013). Unfortunately, the posttranslational modifications have rendered these bacteriocins impossible to chemically synthesize (Nes et al., 2006). These peptides are useful because of their ability to inhibit growth of multiple-drug resistant pathogens and effective use at nanomolar concentrations (Deegan et al., 2010). Class I bacteriocins are small, typically less than 5 kiloDaltons (kDa) (Williams and Chanos, 2012). Subdivisions of class I bacteriocins separate peptides further based on other factors. Members of subdivision Ia are hydrophobic, flexible, pore-forming, cationic, and commonly polycyclic and elongated (Cintas et al., 1998; Nes et al., 2006). The positive charges within the lantibiotics most likely assist with pore-formation, specificially during the initial

interaction and insertion into a net anionic membrane (Deegan et al., 2010). Nisin falls within class Ia. Class Ib bacteriocins are rigid and globular with no net charge (Cleveland et al., 2001; Wieckowicz et al., 2011). These subdivisions are not without classification ambiguities. For example, plantaricin C, produced by *Lactobacillus plantarum*, contains structural elements of both subdivisions in class I while bacteriocin lacticin 3247 contains two peptides, each belonging to the other class of lantibiotics and working in tandem for a greater antimicrobial effect (Nes et al., 2006).

Class II bacteriocins are heat-stable and unmodified except for a disulfide bond between two N-terminal cysteine residues (Cleveland et al., 2001; Wieckowicz et al., 2011). Some other notable features of class II bacteriocins include a small size, typically less than 10 kDa, high glycine content, positive charge, amphiphilic structure, and activity against a wide range of Gram-positive organisms including *L. monocytogenes* (Williams and Chanos, 2012). Class II bacteriocins are further broken up into subclasses IIa, IIb, and occasionally IIc and IId (Cleveland et al., 2001; Wieckowicz et al., 2011). An important aspect of LAB bacteriocins are the IIa peptides, also known as pediocin-like bacteriocins. These bacteriocins that display activity against *L. monocytogenes* have important disulfide bridges between cysteine resides on the N-terminal end of the protein (Cleveland et al., 2001; Wieckowicz et al., 2011), and have a structural identity on the N-terminus consisting of the residues YGNGVxC with x representing any amino acid (Chang et al., 2013; Dirix et al., 2004). Most class IIa bacteriocins permeabilize the membrane of target cells, with the mannose PTS system as a common target (Nes et al., 2006). Himeno et al. (2012) discovered that higher antimicrobial activity was found in bacteriocins with two disulfide bridges than those with only one, likely contributed to by

an increase in stability. The consensus residues found in the N-termini of class IIa bacteriocins should be avoided when designing primers to amplify their genes. Class IIb bacteriocins consist of unmodified two-peptide systems that act synergistically to one another (Nes et al., 2006). Enterocins L50A and L50B as well as enterocins X alpha and beta are examples of two-peptide bacteriocins that have greater activity together than individually (Cintas et al., 1998; Nes et al., 2006). Class IIc and IId bacteriocins comprise categories of leaderless and circular bacteriocins respectively (Nes et al., 2006).

Class III and class IV bacteriocins are less commonly discussed in the literature. Class III bacteriocins are large, usually with an atomic mass of greater than 30 kDa and degrade when heated (Williams and Chanos, 2012). Class IV bacteriocins, such as plantaricin S, form aggregate structures with other molecules like sugars and lipids (Cleveland et al., 2001; Wieckowicz et al., 2011). Due to their large complexes, no class IV bacteriocins have been purified (Cleveland et al., 2001; Wieckowicz et al., 2011).

Overall, the literature is inconsistent with the classification or acknowledgment of existing classes of bacteriocins. Dirix et al. (2004), and Nes and Johnsborg, 2004 only divide bacteriocins into classes I and II with no mention of classes III or IV. Knoll et al. (2008) and Yi et al. (2010) describe bacteriocins in terms of the first three classes. When speaking of classes, a majority of the literature places bacteriocins in four classes although one paper did not mention subclasses (Allison et al., 1994) while the rest did. The inconsistencies of classifying bacteriocins indicate a clear need for sequential classification of these proteins.

Assaying for Bacteriocin Proteins

The classical method for identification of bacteriocin-like peptides is to define their biological and antagonistic activity against an indicator organism and then isolating it and examining the peptide structure (de Jong et al., 2006; Knoll et al., 2008). An example of a common technique is the 'lawn-on-spot' assay in which a small amount of bacteriocin-producing culture, typically 2.5-5 microliters, is put on appropriate media and then overlaid with media containing 0.75% agar and inoculated with 1% of a susceptible indicator bacteria (Allison et al., 1994). LAB commonly produce significant amounts of lactic acid which may mask the antimicrobial activity; however, this can be alleviated by addition of buffer to indicator growth media. In addition, levels of bacteriocin gene transcripts are usually low during early exponential phase and can be up to 4.6 to 7.5 fold higher in late exponential and early stationary phases (Sedgley et al., 2009). Another typical assay for determining bacteriocin activity is the addition of a cell-free supernatant on top of an indicator layer similar to the method previously described – 'spot-on-lawn' (Williams and Chanos, 2012). Williams and Chanos (2012) describe this procedure as misleading because it ignores the possibility that bacteriocins will be unstable after a period of time and a producer might generate more than one kind of bacteriocin which would make the results of the assay ambiguous. Yi et al. (2010) is also critical of this assay, saying that it is not sensitive and requires a lot of time and effort to perform. In addition, some bacteriocins are produced only on solid media with growth temperatures playing a large role in production (Nes et al., 2006). For example, *Enterococcus faecium* L50 produces at least three separate bacteriocins and each has a different optimum temperature showing that antimicrobial activity may fluctuate with growth temperatures

(Nes et al., 2006). Excessive dilution of some Bac+ cultures, for example *Lactobacillus plantarum* C11, can cause loss of their antimicrobial phenotype which will not be regained unless they are exposed to small amounts of spent media (Sedgley et al., 2009). Another functional assay involves quantification of activity by serially diluting bacteriocin-containing supernatants and testing each two-fold dilution against a common indicator typically in a microtiter plate which is analyzed by spectrophotometric interference cause by the growth of indicator organisms (Aymerich et al., 1996; Casaus et al., 1997). Similarly, two-fold dilutions of cell-free supernatents can be plated on top of indicator lawns similar to the 'spot-on-lawn' assay. One final assay for determination of activity involves the separating of proteins on a polyacrylamide gel and overlaying the gel with an indicator layer (Marugg et al., 1992). A common problem with assays that measure activity is the description of that activity. For example, Marugg et al., 1992 describes bacteriocin strength in terms of arbitrary units (AU) which is defined as "5 microliters of the highest dilution of culture supernatant yielding a definite zone of inhibition on the indicator lawn." The definition of AUs differs between literature sources. For example, Aymerich et al., 1996 uses what they call a bacteriocins unit with one of such unit representing the amount of bacteriocins to inhibit growth of an indicator organism by fifty percent; this measure only applies to quantitative growth measurements such as with a microtiter plate assay (Aymerich et al., 1996). Similarly, Cintas et al. (1998) describes their unit, called an antimicrobial unit, as the reciprocal of the highest dilution of the sample causing fifty percent growth inhibition which also must be read spectrophotometrically. Synergistic effects of bacteriocins can similarly only be measured quantitatively and vary based on indicator organism (Cintas et al., 1998). Mass

spectrometer identification can also have errors in results when oxidation of methionine residues and bridges between cysteine residues cause readings to be higher in atomic mass (Casaus et al., 1997). Isolation of novel bacteriocins from cultures can be difficult when screening cultured food samples because culture companies may isolate cultures from their competition's products for use in their fermented or cultured foods (Macwana and Muriana, 2012).

While the aforementioned assays can detect bacteriocinogenic activity of cultures, they do not assess the protein structure or sequence and can provide little help in classifying bacteriocins. In addition, bacteriocin activity is strongly affected by external conditions such as media content and physical parameters like temperature and pH (Cleveland et al., 2001). Traditionally, isolation of protein must first be performed in order to assess structure, and this commonly involves several time-intensive steps. Chang et al. (2013) describe their method to extract and purify a bacteriocin from *Enterococcus faecium* D081821 to include cation-exchange, size-exclusion chromatography, and SDS-PAGE. The class IIa bacteriocin it contained eluted between 0.48M and 0.62M NaCl during cation-exchange, after 12.8 minutes for size-exclusion chromatography, and presented a band between 5 and 10 kDa on an SDS-PAGE gel (Chang et al., 2013). Edman degradation, in which amino acids from proteins are labeled and removed from the N-terminus is commonly used to obtain the amino acid sequence once the peptide has been purified (Casaus et al., 1997). With the advances in PCR amplification and DNA sequencing, much of this work can be accommodated by sequence analysis.

Assaying for Bacteriocin Genes

Recently, researchers have taken to screening genomes for the presence of bacteriocin genes instead of relying on functional assays (de Jong et al., 2006; Knoll et al., 2008). In their research, Wieckowicz et al. (2011) aligned 44 class IIa bacteriocin genes. From these, seven unique, degenerate primer pairs were manually created with the forward primer targeting the conserved amino acid sequence YGNGVxCxxxxC and the reverse primers located within the structural gene (Wieckowicz et al., 2011). They describe the daunting task of creating a set of sufficiently degenerate primers for all sequences as impossible (Wieckowicz et al., 2011). Instead of designed degenerate primers, Macwana and Muriana, 2012 designed a 'PCR primer array' in which 42 structural genes were screened simultaneously with one PCR cycle. Bacteriocin genes can easily be identified in this fashion, although this technique can only target sequences of bacteriocins that are already known and cannot be used to assay genomes for new bacteriocin sequences (Wieckowicz et al., 2011). Macwana and Muriana, 2012 designed their primers using the Primer Express software after obtaining sequences from GenBank. Another possible drawback to this assay is the difference in melting temperatures between the primers which can cause nonspecific amplification if the entire primer array is run simultaneously under the same set of conditions. However, primer design can be optimized to minimize temperature differences. SYBR Green real-time PCR dye was utilized in this assay to detect amplification. Amplimers were sequenced and BLAST was used to assess sequence identity and uniqueness (Macwana and Muriana, 2012). Another genetic approach for using primers is to target conserved areas of bacteriocin genes. Specifically, Yi et al. (2010) used primers which targeted the conserved regions of class IIa

bacteriocins – YGNGV at the N-terminus and LDNAIE located at the C-terminus. They found discrepancies of the sizes of the amplicons which might indicate strain-specific organization of bacteriocin operons (Yi et al., 2010). However, degeneracies of primers can cause large amounts of nonspecific amplification which can lead to problems during sequencing and identification (Wieckowicz et al., 2011). In addition, changing parameters to accommodate for nonspecific amplification has been shown to only reduce amplification of the intended target (Wieckowicz et al., 2011).

In silico Screening of Bacteriocins

Most recently, researchers have utilized mass online accumulations of genome data to artificially screen for bacteriocin genes and bacteriocin gene clusters. One strategy, similar to genetic screening involves searching for common motifs such as the double glycine residue found amongst bacteriocins, but this search technique will only bring up most of the class II bacteriocins and a few class I bacteriocins (Dirix et al., 2004; Nes et al., 2006). Also useful for database searches are the peptide pheromones and unique N-terminal peptidase C39 domain that exists in cognate ABC transporters (Nes et al., 2006). Bacteriocins are screened at the amino acid level but analyzed at the protein level (Dirix et al., 2004). Dirix et al. (2004) utilized the Wise2 program which translates DNA into protein in six different reading frames to scan chromosomes and plasmids for genes. Abi protein genes, a group of metalloproteases found in both eukaryotes and prokaryotes and thought to influence bacteriocin self-immunity, were the target of *in silico* search for bacteriocin genes (Kjos et al., 2010). Genes from the NCBI database were used and

examined for similar structures to bacteriocins (Kjos et al., 2010). Genes were classified as related to bacteriocins when their translated proteins were between 50 and 85 amino acids or they contained a double glycine or *sec*-dependent leader sequences (Kjos et al., 2010).

Bacteriocin Gene Operons

Most bacteriocins exist as one structural gene that encodes for the functional peptide, but their genes are surrounded by other related genes that assist cells in processing and excretion of the peptides. These functional operons commonly contain a structural protein, processing proteins such as cleavage enzymes, transport proteins, and immunity proteins and can be found on any genetic structure in the cells such as chromosomes, plasmids, or transposons (Cleveland et al., 2001; de Jong et al., 2006; Knoll et al., 2008). In particular, pediocin genes *pedABCD* were found on a 9.4 kilobase pair plasmid in *Pediococcus* spp. (Marugg et al., 1992). These gene clusters are preceded by direct repeats between the -40 and -80 positions from the promoter (Sedgley et al., 2009) and is commonly followed by a rho-independent terminator which ends transcription (Casaus et al., 1997). However, these terminators are not found between the genes of the bacteriocin operon which is an indication that the preceding genes are transcribed together (Casaus et al., 1997). An example of a bacteriocin which contains a *rho*-independent stem-loop structure is lactococcin A (Holo et al., 1991). Another common feature that precedes the bacteriocin operon is the double glycine leader sequence previously mentioned (Cleveland et al., 2001) which generates a β-turn in the peptide and allows it to be

cleaved by a protease (Allison et al., 1994). The double glycine motif is structured as LSxxELxxIxGG and is common in bacterial pheromones which are used in quorum sensing activities such as virulence, competence, and production of antimicrobial peptides (Dirix et al., 2004). This consensus sequence may prove to be too short and variable to be used as a target for specific primers. Quorum sensing involves the detection of a constitutively-produced autoinducer by other cells when at high concentrations and causes the induction of other regulatory genes (Dirix et al., 2004). Bacteriocins are produced in this fashion by LAB (Dirix et al., 2004). *Lactobacillus plantarum* C11's production of bacteriocins functions by this mechanism that is very similar to the way virulence is regulated in *Staphylococcus aureus* (Diep et al., 1996). Its bacteriocin is only produced when regulatory peptide, plantaricin A, is in a high enough concentration during the cell's exponential phase (Diep et al., 1996). Cells lacking the ability to produce plantaricin A also lacked any antimicrobial capability (Diep et al., 1996).

Gillor et al. (2008) notes the genetic organization for colicins which contain a toxin gene, a constitutive immunity protein, and a lysis gene. In LAB, regulation often occurs by a two-component system involving a sensing mechanism and a response mechanism, usually via a histidine kinase cascade (Nes et al., 2006). In addition, the operon is regulated by an SOS region upstream of the first gene (Gillor et al., 2008). SOS regions are commonly regulated by a repressor, *LexA*, and an activator, *RecA* (Gillor et al., 2008). While the SOS regulon is common among many *Escherichia coli* genes, it is not commonly found in duplicate like it is among the colicins (Gillor et al., 2008). By contrast, lacticin 481 produced by *Lactococcus lactis* is organized in an operon consisting

of six genes that all are in the same transcriptional direction with no terminator sequences (*lctAMTFEG*) between them (Rince et al., 1997). Rince et al. (1997) describe some of the functions of these genes including antimicrobial activity (*lctA),* amino acid alterations (*lctM*), and an ABC transporter (*lctFEG*).

Bacteriocin Immunity

Another target for genetic approaches to bacteriocin screening is the presence of a bacteriocin immunity gene. Genes within the operon are commonly tested for their ability to provide immunity by cloning into a sensitive organism behind a constitutive promoter and looking for any signs of inhibition (Kjos et al., 2010). For non-lantibiotics the immunity protein will be found downstream of the structural protein; the two are often transcribed together (Casaus et al., 1997). The immunity proteins can exist in several different forms and act by disrupting bacteriocin aggregation at the cell membrane, preventing pore formation, or eliminating the interaction between the bacteriocin and receptor on the membrane (Van Reenen et al., 2006). Hydrophobic regions are common in immunity proteins and may confer ability to anchor themselves into a membrane (Aymerich et al., 1996). Specifically, nisin immunity is caused by a forced sequester of bacteriocins on the cell membrane or active efflux of bacteriocins via ABC transporters (Kjos et al., 2010; Rince et al., 1997). Lactococcin 481 genes *lctFEG*, producing an ABC transporter, provide for self-immunity in *Lactococcus lactis* (Rince et al., 1997). All of the genes must be present in order for the bacterium to be immune; no combinations of two provided immunity (Rince et al., 1997). Abi proteins also provide

immunity to *Lactobacillus sakei* 23K, the genes of which are located downstream of two bacteriocin-like genes (Kjos et al., 2010). For *in silico* screening, Abi proteins could be used to potentially locate nearby bacteriocin genes. The ability to produce gelatinase provides immunity for *Enterococcus faecalis* MC4-1 from its own bacteriocins and bacteriocins from members of the same strain (Sedgley et al., 2009). The gelatinase is thought to degrade the bacteriocins but the absence of immunity during the growth of lawns implies that expression does not happen until MC4-1 enters its stationary phase (Sedgley et al., 2009).

BAGEL: Genome Mining Tool

While open reading frame (ORF) databases exist online, they are not properly annotated and cannot identify genes that are functionally similar but sequentially dissimilar (de Jong et al., 2006). Bacteriocins fall into this category because they are small with poorly-conserved ORF sequences and organization (de Jong et al., 2006). De Jong et al. (2006) explains the potential uses of the BAGEL database for bacteriocin gene detection. BAGEL's main function is to detect and identify bacteriocins and bacteriocin clusters found in the genomic information of microorganisms using information about processing, modification, transport, regulation, and immunity genes (de Jong et al., 2006; Nes et al., 2006). BAGEL uses FASTA, BLAST, hidden Markov models (HMM), Glimmer, RBS finder, Zcurve, and GeneMark along with three ORF prediction tools to scan for ORFs (de Jong et al., 2006). It also uses common motifs (double glycine and FNDLV N-terminal peptide) and adjacent genes (ABC transporters and the C39 family) to scan for

bacteriocin genes (de Jong et al., 2006). BAGEL can also be used to design primers for putative ORFs in search results (Knoll et al., 2008). The database can even be used to detect new bacteriocin genes that have not previously been sequenced.

Enterococcal Bacteriocin Genes

Enterococcus is a genus of lactic acid-producing bacteria that were originally classified under the genus *Streptococcus* until genetic analysis showed enough difference to separate them (Schleifer and Kilpper-Bälz, 1984). Members of *Enterococcus* are Gram-positive, non-sporulating, catalase and oxidase negative, facultative anaerobic, and grow in singlets, pairs, or in chains (Brandão et al., 2010). Enterococci are members of the natural intestinal flora of mammals, including humans, but can also be sources of opportunistic disease (Kurushima et al., 2013; Nes et al., 2006). Nosocomial infections caused by *Enterococcus* spp. include bacteraemia, endocarditis, and urinary tract infections especially in individuals who are immunocompromised (Rehaiem et al., 2014). Still, *Enterococcus* members retain their important lactic acid fermenting abilities which allow them to be used as starter cultures for dairy products (Rehaiem et al., 2014). Some enterococci are thought to be the main fermenting bacteria that give unique flavors to artisanal foods especially near the Mediterranean including cheeses, sausage, and olives (Brandão et al., 2010; Nes et al., 2006). Their ability to survive temporary high temperatures allow them to be useful in this way (Nes et al., 2006). Members of *Enterococcus*, found in food or in other niches, have been found to produce bacteriocins and a large variety of other compounds which give them an environmental advantage

with most bacteriocins being obtained from species *E. faecalis* and *E. faecium* (Nes et al., 2006). Nes et al. (2006) discovered that there is no preferential niche for enterococci that can produce bacteriocins and found 44% of vancomycin resistant enterococci isolated from hospital patients had the ability to produce bacteriocins with the ability to produce three or four different bacteriocins to be a common feature. Bacteriocins from *Enterococcus* spp. may be useful for applications as food preservatives, but virulence factors, such as gelatinase, adhesion to collagen, aggregation substance (asa1), an endocarditis antigen, and occasional ability to be β-hemolytic make them less desirable for addition to foods (Liu et al., 2011).

The bacteriocins of *Enterococcus* species can be divided into 5 groups. Group one contains bacteriocins with antimicrobial activity against a wide variety of Gram-positive organisms but may be β-hemolytic; this group includes the lantibiotic cytolysin (Kurushima et al., 2013). Similarly, members of group two are active against *Streptococcus, Enterococcus faecalis,* and *Staphylococcus aureus*; this group includes enterocin AS-48 and bacteriocin 21 (Kurushima et al., 2013). Group three specifically acts against *E. faecalis, E.hirae, and L. monocytogenes*; a member of this group is bacteriocin 31 (Kurushima et al., 2013). Groups four and five are active only against other members of *Enterococcus,* specifically the species *E. faecalis* and *E. hirae* (Kurushima et al., 2013). Some enterococcins have the ability to inhibit Gram-negative bacteria although this ability is uncommon and usually has a very small range of affected organisms (Liu et al., 2011). For example, enterocins mr10A and mr10B from *E. faecalis* 710C have the ability to inhibit growth of *Brevundimonas diminuta* UFM1, an environmental bacterium without the ability to ferment lactose (Liu et al., 2011).

Most bacteriocins produced by enterococci are plasmid-encoded and belong to the nonlantibiotics (class II) (Nes et al., 2006). Exceptions include cytolysin (class I), a two-peptide lantibiotic produced by *E. faecalis* which displays hemolytic and virulence activity, enterocin AS-48 (class III), and enterolysin A (class IV) (Brandão et al., 2010). Class IIa bacteriocins are perhaps the most relevant with their ability to kill *L. monocytogenes* (Nes et al., 2006). Class IIa enterococins include enterocin A, enterocin P, and hiracin JM79 (Brandão et al., 2010). Interestingly, enterocin A has been shown to have a synergistic effect with class IIc enterocin B (Brandão et al., 2010; Casaus et al., 1997). Enterocin A is encoded on the bacterial chromosome and is different than other class IIa bacteriocins in its N-terminus (Aymerich et al., 1996). Class IIb enterocins require both bacteriocin genes in order to have an effect and an immunity gene encoded nearby (Nes et al., 2006). Enterocins L50A, L50B, mr10A, mr10B, Q, and 1071 fall into this category all with slight homologies to two-peptide bacteriocins in other genera (Brandão et al., 2010; Nes et al., 2006). Enterocins L50A and B are secreted without a leader peptide, co-transcribed without an immunity protein although they are transcribed as an inactive precursor so they are not cytotoxic until secreted, and are similar to staphylococcal hemolysins yet display no hemolytic activity (Cintas et al., 1998). They retain their formylated methionine and do not appear to have any adjacent secretory mechanisms (Cintas et al., 1998). Because of their retained f-Met, these bacteriocins are known as leaderless and cannot easily be detected by *in silico* screening (Nes et al., 2006). Similarly, $BacL_1$ in clinical *Enterococcus faecium* isolates, part of a two-peptide system of what is known as bacteriocin 41, is secreted without processing and is similar

in structure to peptidoglycan hydrolases with its activity being dependent on its partner bacteriocin, BacA (Kurushima et al., 2013).

Brandão et al. (2010) performed an experiment to detect bacteriocin structural genes within 224 *Enterococcus* bacteria of fecal origin. Out of the 224, 102 displayed activity against *L. monocytogenes*, 104 had activity against other indicator organisms, and the remaining 18 had no significant antimicrobial activity (Brandão et al., 2010). In their findings, enterocin P (entP) was the most common bacteriocin found followed by enterocin A (entA) and L50A and L50B (Brandão et al., 2010). In addition, certain bacteriocins were only found from fecal samples of certain sources. Both hiracin JM79 and enterocin B (entB) were detected only from humans and pets and did not come from wild animals (Brandão et al., 2010). Regardless, the presence of enterococcal bacteriocin genes are widespread through all ecological niches and may be useful when isolated, purified, and used as food preservatives even if the cultures may present too much of an infection risk.

Conclusion

The screening of lactic acid bacteria for the ability to produce bacteriocins can be important as the need for anti-pathogenic technology increases with a rapidly growing world population and increased emphasis on food safety. *In silico* screening is frequently used prior to primer design in order to create a specificity of the primer that will apply to the gene and avoid nonspecific amplification with other genes. However, a closer inspection of bacteriocin gene databases shows that many identical or highly similar

bacteriocin genes are given different names by different researchers. Additional consensus is needed in order to adequately catalog discovered bacteriocins. Bacteriocin structural gene sequences are not commonly conserved so a dedicated set of primers could not possibly amplify all potential genes in bacteriocin producers' genomes. Conserved regions within the bacteriocin operon are regular targets for PCR amplification. Primers designed for these conserved regions often contain nonspecific nucleotides to accommodate for the degeneracy of the genetic code which in turn dramatically increases the amount of nonspecific amplification generated by PCR. Since only the structural genes of bacteriocins truly indicate if a strain has the ability to produce these antimicrobial peptides, they are the most logical target for PCR amplification. In order to screen for a large number of sequences, PCR reactions can be run simultaneously with a different set of primers for each reaction. The most common way to do this is to create 96-well plates containing the different reaction mixtures and run all of them in the same cycler. The issue with this approach comes from finding annealing temperatures that work for all primers. Regardless, our aim is to create a multi-reaction PCR plate with primers designed for screening of different enterococcal bacteriocin structural genes simultaneously from pre-screened cultures that display antagonistic ability against *L. monocytogenes*.

CHAPTER II

ISOLATION OF BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA FROM RETAIL FOODS

Abstract

This study examines the presence of bacteriocin-producing (Bac+) lactic acid bacteria (LAB) which can be isolated from food products. Food samples were enriched briefly in MRS broth and plated onto MRSA plates. Antimicrobial activity was determined by an indicator overlay method, using *Listeria monocytogenes* as the primary indicator. Antimicrobial activity was detected in 20 of the 108 different food samples tested with a total number of samples reaching 170. Isolated Bac+ LAB included *Lactococcus lactis, Lactobacillus curvatus, Carnobacterium maltaromaticum, Leuconostoc mesenteroides,* in addition to other Bac+ strains such as *Enterococcus faecium, Serratia plymuthica,* and *Serratia ficaria*. A wide variety of food products contain Bac+ bacteria, and many were isolated from fresh vegetables. These data propose that Bac+ LAB are widely dispersed as part of the natural flora of unprocessed foods.

Introduction

Considering consumer's interest in natural products and the high cost of foodborne illness, food producers look for new ways to preserve food. The development of pathogenic strains with

increasing resistance to antibiotics is another concern the food industry is handling (Nes et al., 2006). Numerous papers have been published discussion and characterizing the use of ribosomally synthesized antimicrobial peptides, termed bacteriocins, as one possible way to replace chemical methods of preservation (Casaus et al., 1997; Macwana and Muriana, 2012).

Bacteriocins are produced naturally in foods, and often bacteriocin-producing bacteria are added as started cultures to fermented foods widely sold in the marketplace (Nes et al., 2006; Nes and Johnsborg, 2004). Not only do bacteriocins display antagonistic activity towards foodborne pathogens, but they also show activity against spoilage organisms (Knoll et al., 2008; Macwana and Muriana, 2012). Bacteriocins have varying inhibitory spectra, but provide a competitive and protective role against others species to which they are closely related (de Jong et al., 2006; Knoll et al., 2008). Bacteriocins can be used in tandem with other antimicrobial treatments in what is known as the hurdle effect in order to enhance the preservation of food (Cleveland et al., 2001). For example, the antimicrobial effect of nisin is enhanced when mixed with a metal chelating molecule like EDTA or a pulsed electric field which can also increase its effectiveness against Gram-negative bacteria due to temporary pores being formed in their outer membrane (Cleveland et al., 2001).

Lactic acid bacteria (LAB) are a major grouping of bacteria that have been shown to produce bacteriocins. LAB are Gram-positive and considered "generally recognized as safe" (GRAS) by the United States Food and Drug Administration (FDA); however, their bacteriocins are not automatically granted that status (Macwana and Muriana, 2012; Nes and Johnsborg, 2004). LAB such as *Lactobacillus* spp. are also well documented to have a probiotic effect when ingested from food (Casaus et al., 1997). Typically, bacteriocins produced by LAB will only be effective against Gram-positive bacteria, which includes *L. monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum*, because Gram-negative bacteria are protected against most of their activity by the outer membrane (Liu et al., 2011).

Bacteriocins are typically categorized based on their amino acid sequence, composition, and inhibitory spectra. Bacteriocins commonly include cationic charges, amphiphilic conformations, and interactions with target membranes (Knoll et al., 2008). Bacteriocins are divided into classes based upon their common features including conserved biochemical and structural properties although some features cause them to be classified incongruently by different researchers. Class I bacteriocins, known as lantibiotics, are unique because of the presence of unusual amino acids in their structures including lanthionine, methyl-lanthionine, dehydrobutyrine, and dehydroalanine and are small, typically fewer than 5 kDa (Cleveland et al., 2001; Wieckowicz et al., 2011; Williams and Chanos, 2012). Class II bacteriocins consist of unmodified peptides that are heat-stable, small, and have high glycine content (Wieckowicz et al., 2011; Williams and Chanos, 2012). This group of bacteriocins is known for their wide range of activity against Gram-positive organisms including *L. monocytogenes* (Williams and Chanos, 2012). Class III and IV bacteriocins are less commonly featured in studies. Both are large, with class IV bacteriocins forming large complexes with sugars and lipids and being difficult to purify (Cleveland et al., 2001; Wieckowicz et al., 2011).

Materials and Methods

Bacterial Cultures, Growth Conditions, and Storage

Cultures of the Bac+ LAB were stored after isolation by inoculating a colony into a test tube containing 9 mL of MRS broth and incubated overnight (12-16 hours) at 30^{o}C. Master cultures were created by centrifuging overnight cultures at 6000 RPM for 15 minutes, decanting the spent media from the LAB pellet, and resuspending the pellet in 2 mL of milk-based freezing media (11% non-fat dry milk powder, 1% glucose, 0.2% yeast extract). Resuspended cultures were then aliquoted into 8 mL storage vials and stored at -80^{o}C. *Listeria monocytogenes* 39-2 was grown in tryptic soy broth and frozen master stock cultures were prepared as described above.

Isolation of Bacteriocin-Producing (Bac+) LAB from Food

Food items were obtained from multiple stores in Stillwater, OK including Walmart, Food Pyramid, Crêpe Myrtle, and Consumers. These consisted of raw meat, fruit, vegetables, and herbs; processed foods were avoided. Food samples were cut (if too large) and enriched in Whirl-Pak® filter bags (Nasco, Fort Atkinson, WI) as a 10 fold weight/volume dilution in MRS Broth (Difco Laboratories, Detroit, MI) for 24 h at 30^{o}C. Newer food samples were enriched for 4 h after isolation resulted in mostly bacteria of one genus and species. Enriched broth was serially diluted and spread plate onto buffered (0.1 M sodium phosphate dibasic and 8 mM sodium phosphate monobasic) MRSA plates (Fig. 1). Plates were allowed to dry before being covered in a thin layer of 1.5% agar MRSA (the sandwich layer). Colonies sandwiched between the layers were allowed to grow until pinpoint or slightly larger in size, anywhere from 12 to 48 h. Once colonies had grown, molten 0.75% agar MRSA agar was inoculated with 1 ml of indicator organism (e.g. Listeria monocytogenes) per 100 mL of molten media and poured over the top of the sandwich layers. Plates were returned to incubate at 37oC overnight. Plates were checked for zones of inhibition and food isolate colonies were excised from between the sandwich and base layer by flipping the plate over and cutting through the base layer (Figs. 2 & 3). Colonies were subsequently quadrant streaked, patch plated, and spotted under a lawn of indicator organism to ensure isolation of bacterial colonies from each other.

Extraction of Total Bacterial DNA

Prior to extraction of DNA, overnight copies of the Bac+ isolates were inoculated into tubes containing MRS broth and grown at 30^{o}C. 1 mL of overnight culture was transferred into sterile, 1.5 mL microcentrifuge tubes and spun down in a centrifuge at 12,000xg for 1 minute. Resulting supernatant was discarded, and pellets were washed twice in 0.5 mL of sterile, deionized water. The final pellet was resuspended in 100 μL pH 7.4 10 μM Tris. Pellets resuspended in Tris were

transferred to new microcentrifuge tubes containing acid washed silica beads (VWR International, LLC, Radnor, PA) so that the liquid covers the top of the beads. Bead-pellet mixtures were then put on ice for 3 minutes and subsequently transferred to a pulsing vortex with attached shaker head for 3 minutes. After another cycle of chilling and shaking, samples were placed on ice for 4 minutes. Tubes containing the beads and sheared cells were placed into a centrifuge and spun down at 12,000 xg for 2 minutes. 50-100 μL of supernatant containing extracted DNA was transferred from the top of the beads to a new microcentrifuge tube and then stored at -20^{o}C until used further. Concentration of DNA was then measured using a NanoDrop® ND-1000 spectrophotometer.

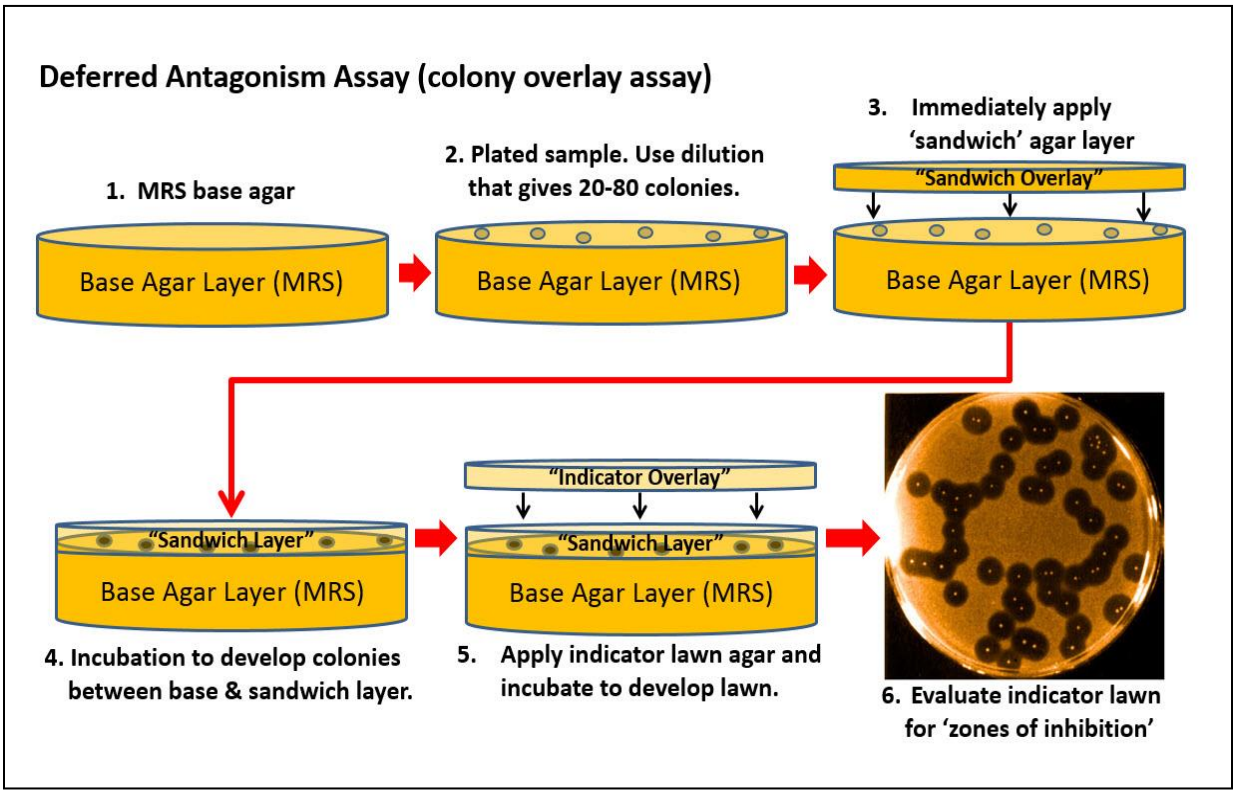

Figure 1. Colony overlay assay (deferred antagonism) to identify bacteriocin-producing (Bac^{+}) isolates.

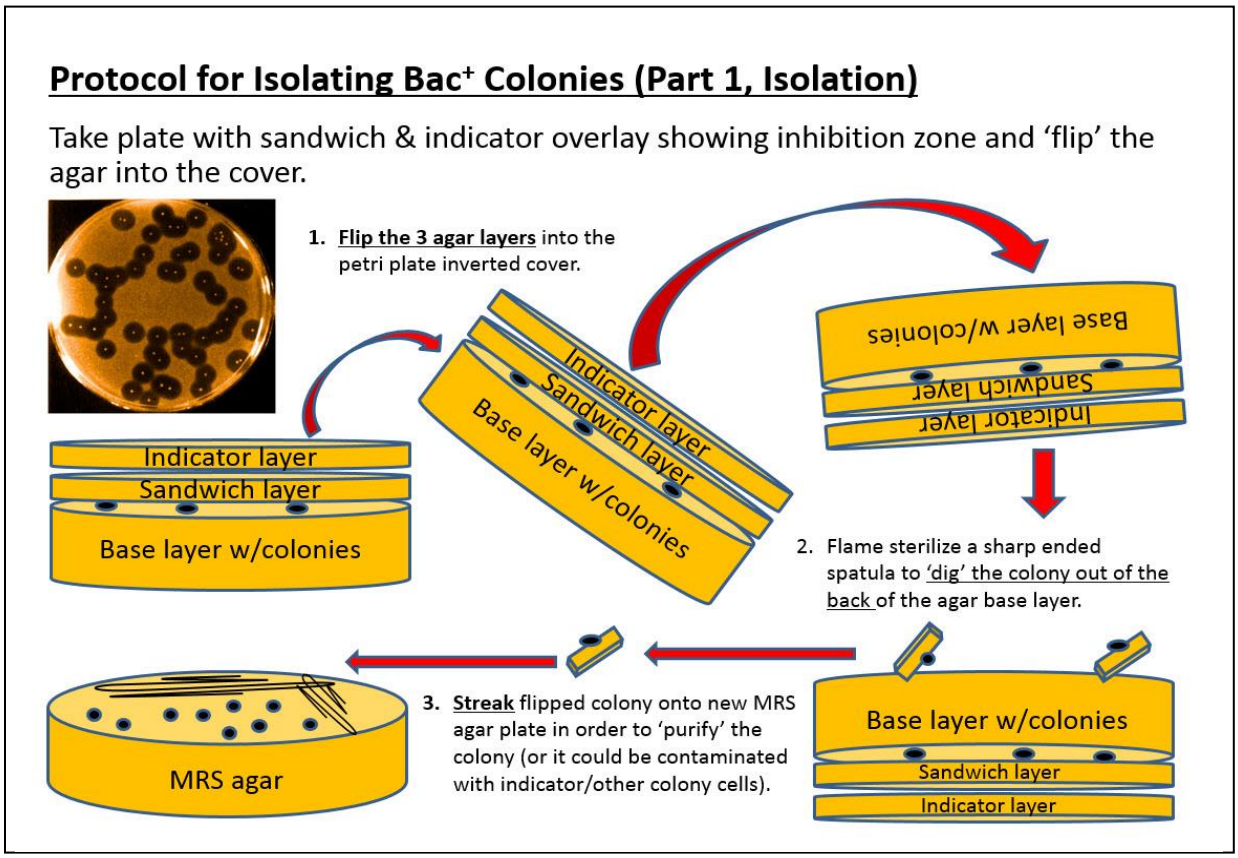

Figure 2. Isolation and retrieval of Bac+ colonies from 'sandwich overlay' plates.

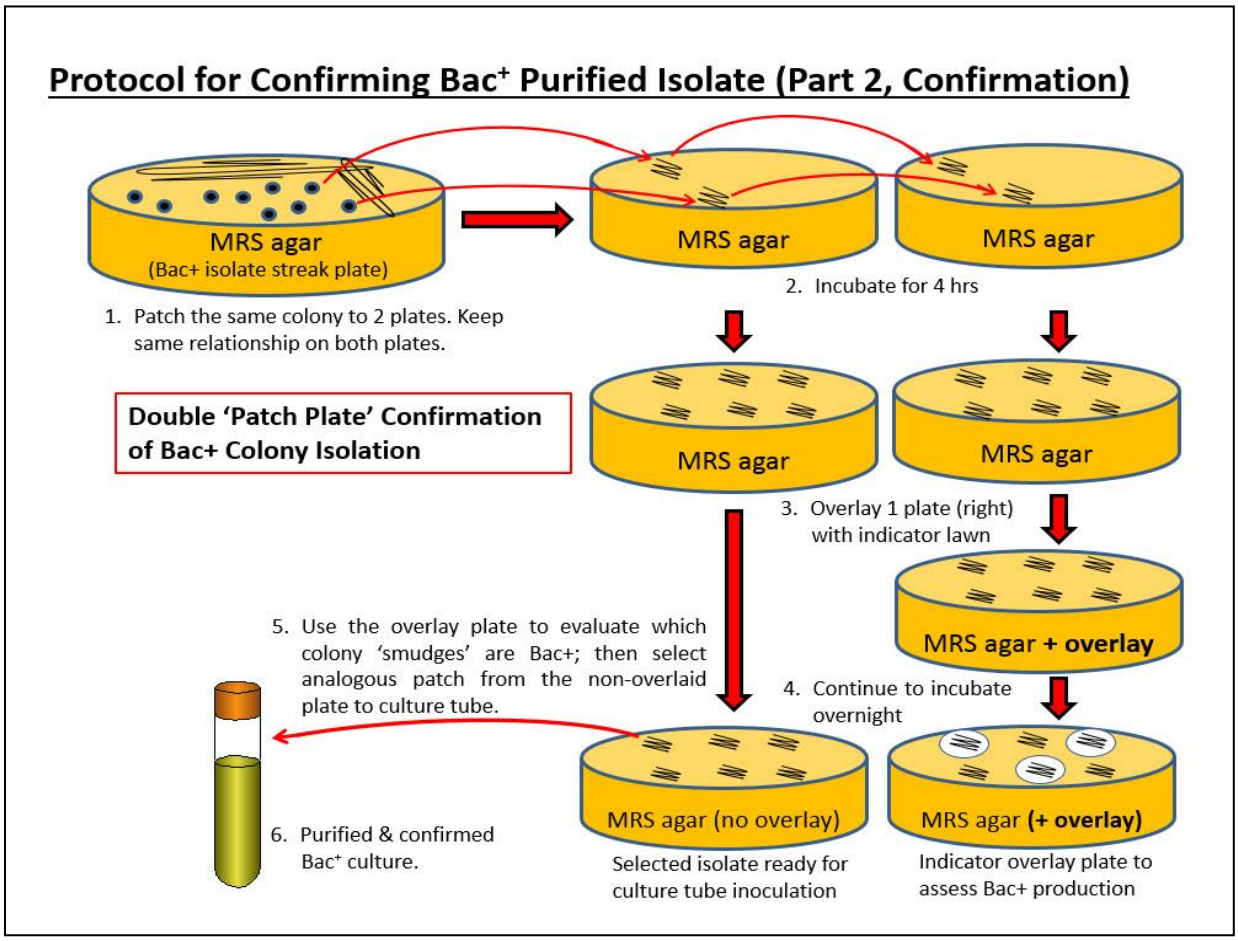

Figure 3. Confirmation of Bac$^+$ phenotype from isolated Bac$^+$ colonies.

Identification of Isolates by 16S rRNA Gene Amplification, Sequencing, and Analysis

The amplification of 75 Bac+ isolates was assessed using PCR with universal 16S ribosomal RNA primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 1391R (5'-GACGGGCGGTGTGTRCA-3') described by Turner et al. (1999). The PCR reaction mix consisted of the following:

- 1 μL total DNA extract (obtained as previously described)
- 5 μL of 5X GoTaq® PCR Buffer (Promega, Madison, WI)
- 2.5 μL of 15 mM $MgCl_2$ solution
- 2 μL of 5 mM dNTP mix
- 1.25 μL of each primer at 10 μM (515F and 1391R)
- 0.25 μL of 5 U/μL GoTaq® Flexi DNA Polymerase (Promega)

The final concentration of primers used was 500 nM in a final volume of 25 μL for each reaction. Reaction mixtures were placed into 0.2 mL PCR tubes and then subjected to thermal cycling using a PTC-200 Peltier Thermal Cycler (MJ Research, St. Bruno (Quebec), Canada) with the following thermal cycles:

- initial denaturation at 95^{o}C for 4 min
- 30 cycles of 94^{o}C for 1 min (denaturation), 60^{o}C for 45 s (annealing), and 72^{o}C for 1 min (extension)
- final extension cycle at 72^{o}C for 4 min
- final hold at 4^{o}C

All PCR reactions were run with a negative control (no added template DNA) and a positive control (using template DNA from previous runs). PCR reactions were accompanied by agarose gel electrophoresis and DNA sequence analysis in both directions.

Amplimers of 16S rRNA genes were purified via the GenCatch™ Advanced PCR Extraction Kit (Epoch Life Sciences, Missouri City, TX). The entire PCR reaction was transferred and mixed with 500 µL of supplied PX Buffer in a sterile microcentrifuge tube. Buffered PCR reaction mixtures were transferred to the top of Spin Columns which rested in provided collection tubes. The mixture was centrifuged at 5000xg for 1 minute, and the filtrate was discarded. The DNA retained by the Spin Columns was washed with WN Buffer and then WS buffer by adding 500µL to the column and centrifuging at 5000xg for 1 minute. The filtrate was again discarded between and after washing. Washed columns were spun down at 13,000xg for 3 minutes to dry. Dried columns were placed into new microcentrifuge tubes and 25 µL of Elution Buffer was added to the center of the column membranes and allowed to sit at room temperature for 3 minutes. Eluted DNA was collected by centrifuging the column at 13,000xg for 2 minutes and stored at -20^{o}C until used.

Purified DNA was submitted to the Dept. of Biochemistry and Molecular Biology Recombinant DNA/Protein Resource Facility (Oklahoma State University) using an automated DNA sequencer via "BigDye™"-terminated reactions analyzed on an ABI Model 3700 DNA Analyzer. ABI sequence files were analyzed using MEGA5 by cutting out 5' and 3' regions of high background noise. Both forward and reverse sequences were compared and aligned in order to increase accuracy. Consensus sequences between the forward and reverse amplimers were analyzed using NCBI's Nucleotide BLAST. Identities were recorded, and sequences are compiled together and aligned to create a maximum likelihood tree.

Results

In this study, our objective was to identify bacteriocinogenic LAB indigenous to raw and fresh retail foods. We isolated Bac+ LAB from 23 food samples out of 170 total samples (13.5% occurrence). This number falls slightly below the 21% isolation rate found previously when

enriching food samples before plating (Garver and Muriana 1993). We isolated a total of 43 Bac+ LAB colonies from the 23 food samples where we occasionally found two or three Bac+ organisms from a single food sample. Isolates were identified by utilizing universal 16S ribosomal RNA primers in PCR reactions with extracted total DNA. Our results revealed 43 isolates spanning seven different genera (**Table 1**) including *Lactococcus lactis* (24, 55.8%), *Carnobacterium maltaromaticum* (8, 18.6%), *Enterococcus faecium* (5, 11.6%), *Lactobacillus curvatus* (3, 6.98%), *Leuconostoc mesenteroides* (1, 2.3%), *Serratia plymuthica* (1, 2.3%), and *Serratia ficaria* (1, 2.3%). The phylogenetic distribution is shown in **Figure 5** as a Maximum Likelihood Tree constructed using the MEGA 5 genetic analysis software.

During our analysis, we observed a much higher incidence rate of *Lactococcus lactis* (24) than any other isolate. This number was threefold the occurrence of the next most frequent organism, *Carnobacterium maltaromaticum* (8). *Lactococcus lactis* isolates were obtained from green beans (GBN), radish (RD, RDSH), sweet potato (SP), yellow onion (YO), shredded lettuce (SL), jalapeno peppers (PJP), asparagus (ASPG), whole lettuce (FL), and bean sprouts (BSP). *Lactobacillus curvatus* isolates were obtained only from ground beef (BEEF) and the sole *Leuconostoc mesenteroides* isolate from breakfast sausage (BFS). *Carnobacterium maltaromaticum* isolates were obtained from ground beef (LGBF, GBF), collard greens (COG), chicken wings (CHW), tofu (TOF), ground pork (GPK), and ground Angus chuck (GAC). *Enterococcus faecium* isolates were obtained from thyme (THYME) and pork sausage (GPK). The *Serratia plymuthica* isolate was obtained from russet potatoes (POT) while the *Serratia ficaria* isolate was obtained from Chinese celery (CCEL). Although 25 samples were tested, no Bac+ LAB were isolated from fresh fruit

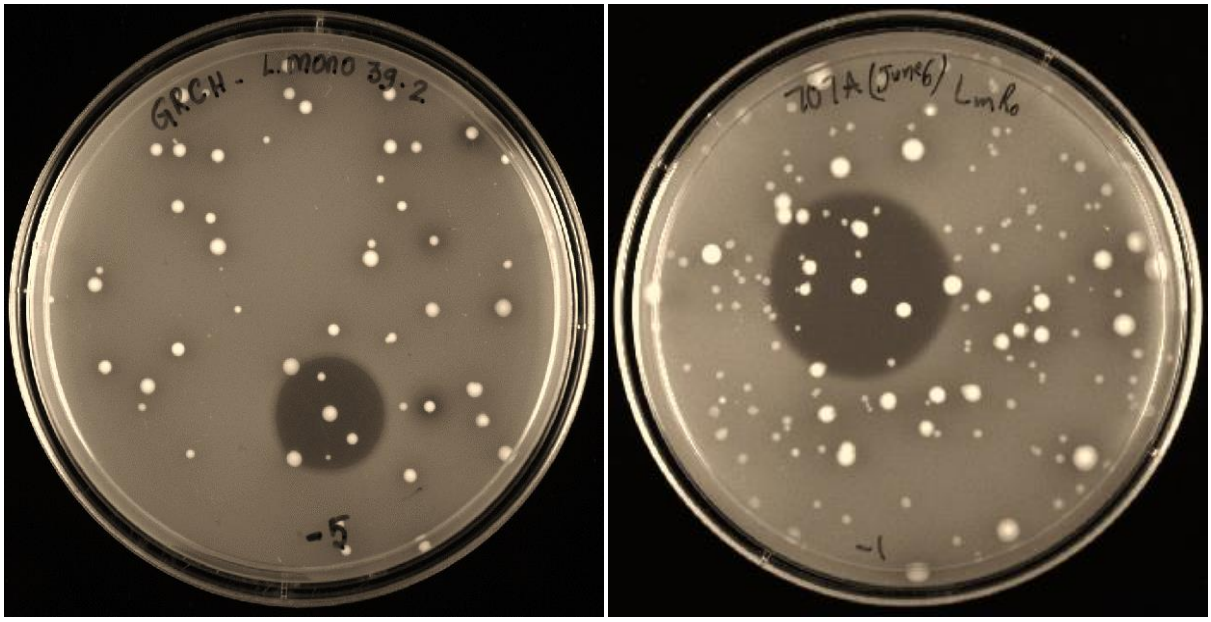

Figure 4. Representative bacteriocin inhibition zones obtained after *Listeria monocytogenes* indicator agar was overlaid onto 'sandwiched' colonies plated from enriched food samples. The Bac+ colonies were then isolated/purified as indicated above, identified by 16S rRNA sequence analysis, and characterized for potential use as food preservatives.

Table 1 – Food samples and numbers and identities of isolated Bac+ microorganisms.

	Food Sample	Samples with Bac+ LAB	Total # Samples	Genus/Species
Meat and Proteins	Ground Beef	2	6	*Lactobacillus curvatus* BEEF 2L-1/2L-2/3 *Carnobacterium maltaromaticum* LGBF-1, GBF-1
	Pork Sausage	2	10	*Leuconostoc mesenteroides* BFS-1 *Enterococcus faecium* JCP-9/B-5/M-2
	Tofu	1	2	*Carnobacterium maltaromaticum* TOF-1
	Chicken Winglets	1	1	*Carnobacterium maltaromaticum* CHW-1
	Ground Pork	1	3	*Carnobacterium maltaromaticum* GPK-1
	Ground Angus Chuck	1	1	*Carnobacterium maltaromaticum* GAC-1/2
	Ground Turkey	0	5	
	Beef Roast	0	2	
	Honey Ham	0	1	
	Pork and Chicken Brats	0	2	
	Pork (for stew)	0	1	
	Chicken Gizzards and Hearts	0	1	
	Hot Dogs	0	1	
	Cubed Ham	0	2	
	Cube Steak	0	1	
	Shredded Ham	0	1	
	Beef Tripe	0	1	
	Chicken Breast	0	1	
	Ground Chicken	0	1	
	Pork Meatball	0	1	
	Chicken Feet	0	1	
Vegetables	Green Lettuce (Whole)	1	2	*Lactococcus lactis* FL-1/2/M-1/S-2
	Asparagus	1	2	*Lactococcus lactis* ASPG-1/2/3
	Iceberg Lettuce (Shredded)	1	2	*Lactococcus lactis* SL-1/2/3
	Yellow Onion	1	1	*Lactococcus lactis* YO-1/2/3
	Raddish (Red)	2	6	*Lactococcus lactis* RDSH-1/2/3, RD
	Poblano/Jalapeno Mix	1	1	*Lactococcus lactis* PJP-1

	Sweet Potato	1	3	*Lactococcus lactis* SP-1/2
	Bean Sprouts	1	2	*Lactococcus lactis* BSP
	Collard Greens	2	2	*Carnobacterium maltaromaticum* COG-1
	Russet Potato	1	2	*Serratia plymuthica* POT-1
	Green Beans	1	2	*Lactococcus lactis* GBN-1/2/3
	Chinese Celery	1	1	*Serratia ficaria* CCEL-1
	Green Onion	0	4	
	Celery	0	2	
	Anaheim/Jalapeno Mix	0	1	
	Broccoli	0	2	
	Fajita Veggie Mix	0	1	
	Jalapeno	0	3	
	Red Leaf Lettuce (Whole)	0	3	
	Anaheim Pepper	0	1	
	Bell Pepper	0	3	
	Poblano Pepper	0	1	
	Habanero Pepper	0	1	
	Kimchi	0	1	
	White Mushroom (Whole)	0	1	
	Sugar Snap Peas	0	1	
	Parsnip	0	3	
	Portabella Mushroom (Whole)	0	1	
	Artichoke	0	1	
	Kale	0	1	
	Green Cabbage (Whole)	0	1	
	Carrot (Whole)	0	1	
	Mustard Greens	0	1	
	Leeks	0	1	
	Tomatillo	0	1	
	Crook Neck Squash	0	1	
	Spinach (Whole)	0	2	
	Shallots	0	1	
	Yam	0	1	
	Taro	0	1	
	Japanese Sweet Potato	0	1	
	Cauliflower	0	1	
	Yu Choy	0	1	
	Avocado	0	1	
	Turnip	0	1	
	Corn (on the Cob)	0	1	
	Cucumber (Whole)	0	1	
	Burdock (Whole)	0	1	
	Bok Choy	0	1	

	Water Chesnut	0	1	
	Egg Plant	0	1	
Dairy	Colby Jack Monterey	0	1	
	Sheep Cheese	0	1	
	Provolone Cheese	0	1	
Fruit	Cantaloupe	0	2	
	Cherry	0	2	
	Strawberry	0	2	
	Lemon	0	1	
	Lime	0	1	
	Orange	0	2	
	Kiwi	0	1	
	Mango	0	2	
	Blueberries	0	1	
	Blackberries	0	1	
	Red Grapes	0	1	
	Banana	0	1	
	Honeydew	0	1	
	Peach	0	1	
	Asian Pear	0	1	
	Green Plantain	0	1	
	Plum	0	1	
	Nectarine	0	1	
	Grapefruit	0	1	
	Coconut	0	1	
Herbs	Thyme	1	1	*Enterococcus faecium* THYME 2/3
	Cilantro	0	3	
	Baby Dill	0	1	
	Mint	0	1	
	Rosemary	0	1	
	Sage	0	1	
	Parsley	0	3	
	White Garlic	0	1	
	Ginger	0	1	
	Lemon Grass	0	1	
	Basil	0	1	
	Horseraddish	0	1	
	Garlic	0	2	

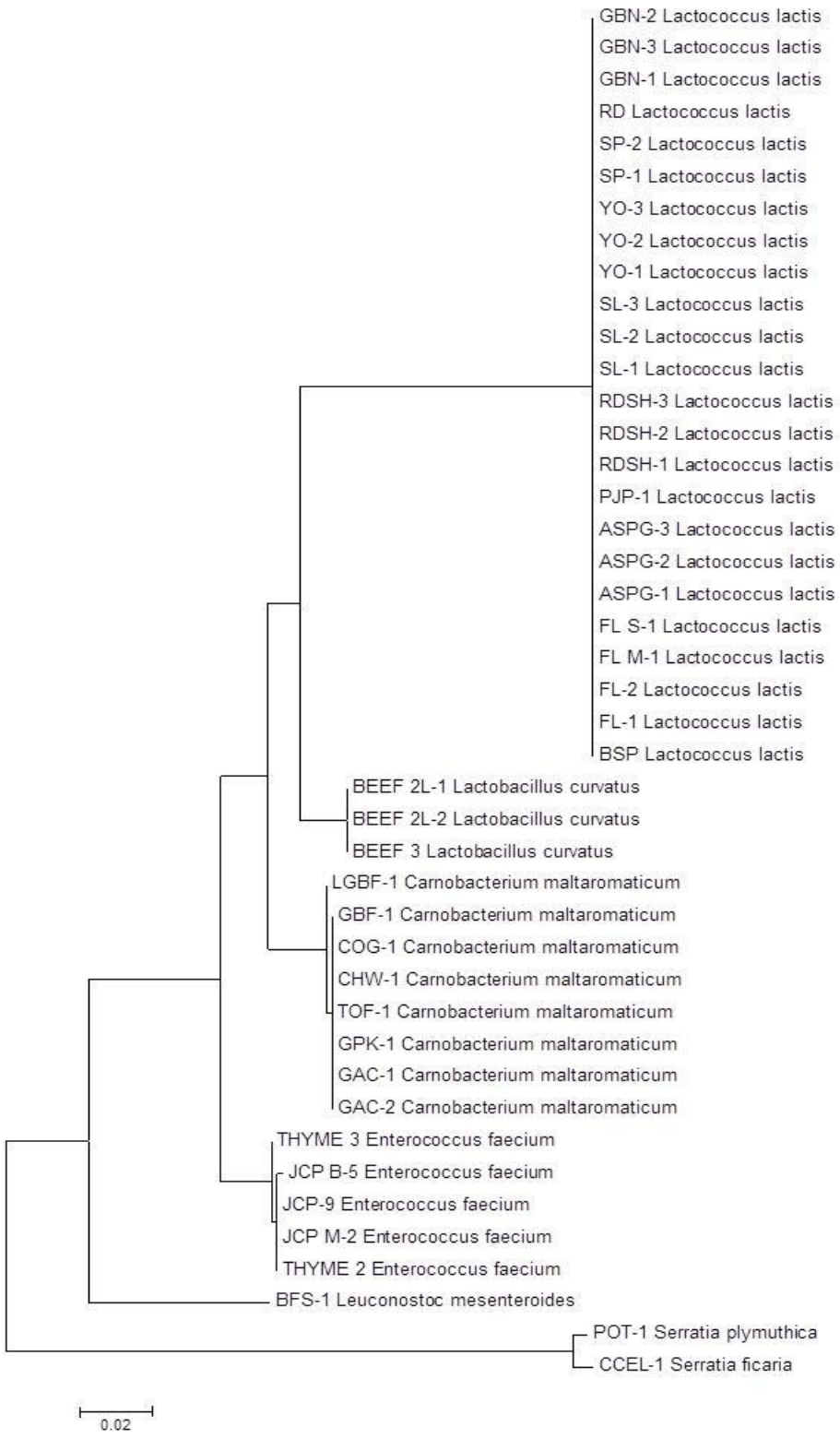

Figure 5. Maximum likelihood tree for 16S rRNA sequences of food-isolated Bac+ LAB

Discussion

In recent decades, interest in bacteriocins, especially those of lactic acid bacteria, has increased due to their ability to inhibit foodborne pathogens and spoilage bacteria. Additionally, bacteriocins may be able to replace some chemical preservatives and provide a more natural preservative ingredient as requested by consumer demand. Many of these Bac+ bacteria are already found in foods available on the market, whether added intentionally or as part of the natural flora. Many Bac+ lactic acid bacteria are added as starter cultures to fermented foods alternately consumed as probiotics (Casaus et al., 1997).

Microbiological analysis of common, unprocessed market foods carried out in this study implicates a variety of bacterial genera with the ability to produce bacteriocins including some that are not members of the lactic acid bacteria. The difference in numbers of isolates that are not LAB are not indicative of their relative presence on these food products. Rather, the use of MRS media, selective for lactic acid bacteria, limits the recovery to organisms that can grow on it. In addition, multiple samples of the same food product revealed an inconsistent presence of Bac+ bacteria upon repeated testing.

We observed differences in the identities of LAB isolates upon altering enrichment time of food samples. Initially, samples were incubated for 24 hours in MRS before plating. Isolates gathered from this technique were mainly *Lactococcus lactis* and included isolates designated ASPG, RDSH, PJP, SL, SP, and YO. Due to the high prevalence of *Lactococcus lactis*, we recommend reducing enrichment times to 4 hours in order to isolate the full array of other genera that may be present.

According to our results, *Lactococcus lactis* was the most common Bac+ LAB isolated from foods followed by *Carnobacterium maltaromaticum*. This may be explained by our initial

extended enrichment process when first collecting samples, providing the quickest-growing bacteria (or bacterium that can grow to the highest numbers) with an advantage during the final screening via plating to extinction and overlay with indicator. Regardless, all isolates we obtained have been documented with the ability to produce bacteriocins (Aymerich et al., 1996; Diep et al., 1996; Foulds and Shemin, 1969; Holo et al., 1991; Martin-Visscher et al., 2008; Stiles, 1994).

This work extends our knowledge of the ubiquitous distribution of Bac+ bacteria in foods. The extent of this distribution allows for researchers to isolate Bac+ bacteria readily from available market sources. This information may assist future work on isolating natural sources of antimicrobials that may be used against foodborne pathogens including *Listeria monocytogenes*.

CHAPTER III

SCREENING OF BACTERIOCIN STRUCTURAL GENES IN *ENTEROCOCCUS* SPP. USING A PCR PRIMER ARRAY

Abstract

Twenty-two bacteriocin-producing *Enterococcus* isolates obtained from food and animal sources were screened for bacteriocin structural genes using a PCR primer array based on enterococcal bacteriocin gene sequences in the NCBI GenBank database. Isolates screened included members from the following species: *E. durans* (1), *faecalis* (4), *faecium* (12), *hirae* (3), and *thailandicus* (2). Each isolate contained at least one of the screened structural genes. Fifteen of the twenty-two isolates yielded at least two different bacteriocin genes. Enterocin A (entA), enterocins mr10A and mr10B (mr10AB), and bacteriocin T8 (bacA) were the most commonly found structural genes in order of decreasing prevalence. Our results confirm that enterococci display a high degree of bacteriocinogenic potential which may play promising a part in biopreservation of food.

Introduction

Chemical treatments may be able to control the growth of foodborne pathogens in food, but with growing consumer demand for natural products and ingredients and an increasing resistance to the use of antibiotics in animal feed, a new solution is needed. Bacteriocins are ribosomally synthesized peptides produced by bacteria capable of killing other bacteria by forming pores in the target membrane and lysing the cell (Casaus et al., 1997; Macwana and Muriana, 2012). Bacteriocins are supposedly most effective against bacteria that are closely related (Cleveland et

al., 2001) but this doesn't always hold true. Of particular interest among the bacteria which produce bacteriocins, are lactic acid bacteria (LAB). LAB are considered "generally recognized as safe" (GRAS) by the United States Food and Drug Administration (FDA) as food ingredients, including, bacteriocins produced by LAB in cultured or fermented foods (Macwana and Muriana, 2012; Nes and Johnsborg, 2004). Some LAB also produce other inhibitory compounds such as reuterin, terutericyclin, hydrogen peroxide, and lactic acid (Nes and Johnsborg, 2004).

Bacteriocins are typically divided into a number of classes and subclasses based on physiological properties and activity; however, the number of acknowledged classes varies between publications. While homology, size, and isoelectric point are common indicators used to classify bacteriocins, the current method of classifying and confirming novel bacteriocins relies on amino acid and gene sequences (Chang et al., 2013; Zouhir et al., 2010). Bacteriocins are also given different names by researchers who discover the same bacteriocins at different times. Of 107 bacteriocins, 40 fell into one class and subclass while 20 fell into more than one class and subclass at the same time (Zouhir et al., 2010).

Enterococcus is a genus in the order *Lactobacillales* in which members produce lactic acid and many have also been documented to produce bacteriocins (Kurushima et al., 2013; Liu et al., 2011; Nes et al., 2006). Members are Gram-positive , non-sporulating, catalase and oxidase negative, facultative anaerobic, and grow in singlets, pairs, or in chains (Brandão et al., 2010). Their ability to ferment sugars into lactic acid makes them important for starter cultures in fermented ('artisanal') dairy products. Enterococci have the ability to produce a number of inhibitory compounds with simultaneous production of three to four bacteriocins being a common feature (Nes et al., 2006). In a study of the distribution of enterococcal bacteriocin genes among clinical isolates, enterocin P was the most widely distributed bacteriocin followed by enterocin A, and enterocins L50A and L50B (Brandão et al., 2010). Unfortunately, *Enterococcus* spp. also exhibit a number of virulence factors including gelatinase, adhesion to collagen, aggregation

substance (asa1), an endocarditis antigen, and β-hemolytic substances which make them less likely candidates for being directly added to food (Liu et al., 2011).

Materials and Methods

Bacterial Strains, Storage, and Growth Conditions

This study included 22 isolates from the *Enterococcus* genus obtained from food, rumen samples, and fecal samples. Within the 22, 12 belonged to *E. faecium,* 4 to *E. faecalis,* 3 to *E. hirae*, 2 to *E. thailandicus,* and 1 to *E. durans*. The isolation and detection of antimicrobial activity was performed as described on page 26. Frozen master cultures of enterococcal strains were stored in milk-freeze media at -80^{o}C, and 100 µL of master culture was added to MRS broth at 30^{o}C for 12-16 hours prior to use.

Primer Creation

Bacteriocin genes were found using the online database Bactibase (http://bactibase.pfba-lab-tun.org/main.php) and searching for individual gene sequences produced by members of the genus, *Enterococcus*. Duplicates and highly homologous gene sequences were condensed into a single primer set. Gene selections were made using bacteriocin structural genes and neighboring immunity proteins. Adjacent ABC transporters and other similar features were excluded due to high homology between genes. Primers were designed from gene sequences using the online Primer3 software (http://simgene.com/Primer3). Once created, primers were analyzed against each bacteriocin gene using the MEGA 5.2 software (http://www.megasoftware.net/) to ensure cross-amplification would not occur. Primers were ordered from Integrated DNA Technologies (IDT, Coralville, IA).

PCR Detection of Enterococcal Bacteriocin Genes

The presence of bacteriocin related genes was determined by PCR. Initially, total bacterial DNA was isolated using the BAX® protease lysis method (DuPont Qualicon, Wilmington, DE). Overnight culture (5 uL) was lysed in 200 uL protease mixture at 55°C for 60 minutes followed by a deactivation step at 95°C for 10 minutes. PCR amplification was performed on a DNA Engine Opticon 2 (MJ Research, St. Bruno, Quebec, Canada) in 25 uL reaction mixtures. Each reaction contained iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA), a final concentration of 60 nM for each primer, and 5 uL of the cell lysate diluted 1:5. The cycling program was preceded by an initial denaturation at 95°C for 15 minutes. The specific cycling parameters consist of 40 cycles of the following denaturation at 95°C for 15 seconds, annealing at 60°C for 60 seconds, and elongation at 72°C for 60 seconds followed by a plate read. PCR products were verified by melting curve analysis (50°C to 90°C with a read every 0.2°C and hold for 0.02 seconds) and electrophoresis on a 2% (wt/vol) agarose (FMC Corporation, Philadelphia, PA) gel at 80V for 1h, using a 100 bp ladder for size verification and viewed using the ChemiDoc™ XRS System UV transilluminator (Bio-Rad). PCR products were purified using the GenCatch™ Advanced PCR Extraction Kit (Epoch Life Science, Missouri City, TX) and submitted for sequencing to the Oklahoma State University Recombinant DNA and Protein Core Facility.

Results

Enterococcus spp. are commonly found among other lactic acid bacteria in food and the environment. Enterococci have been documented to produce bacteriocins which may give them an environmental advantage (Nes et al., 2006). The ability to produce several bacteriocins is also a common feature seen among these lactic acid bacteria (Nes et al., 2006). Bacteriocin-producing strains of *Enterococcus* have been isolated from fermented foods (Rehaiem et al., 2014), the

intestinal flora of mammals including humans (Kurushima et al., 2013), and retail meat (Garver and Muriana, 1993).

Primer Design

We found 37 *Enterococcus* bacteriocin DNA sequences in the Bactibase database and used them for creating primers. Aligning the sequences in the database revealed that genes with identical sequences were denoted as different bacteriocins with different accession numbers. Upon removal of highly homologous sequences, the DNA sequences were condensed to 16 unique sequences listed in **Table 2** with highly homologous sequence names under the heading "Homologous Genes." Primary names for the primer pairs created with homologous genes were chosen subjectively. **Figure 8** shows a maximum likelihood tree, created with the MEGA 5.2 software, of the bacteriocin genes used in this study.

PCR Detection of Enterococcal Bacteriocin Genes

The 22 *Enterococcus* isolates used in this study possessed the ability to inhibit growth of *Listeria monocytogenes* and contain at least one bacteriocin gene. Gene presence is shown in **Table 3** along with homology to the documented gene sequence listed in **Table 2**. In this study, the gene for enterocin A (entA) occurred most frequently (77.3%) with at least one isolate in each species showing amplification for this gene. The amplification using the mr10AB primers was the next most frequently detected bacteriocin (63.6%). Homology relatedness values for these amplicons with various *Enterococcus* bacteriocins are presented in **Table 3**. Sequences obtained from bacteriocin mr10AB primers were all more homologous to L50A and L50B enterococcin sequences in the GenBank database. L50A/L50B and mr10AB are highly homologous (>95%) and were considered interchangeable in this study. Other percentages for amplification of specific structural genes from among our isolates are bacA (31.8%), enxAB (18.2%), entP

(18.2%), entB (9.1%), and munA (9.1%). Structural genes for avicin A (avcA), columbicin A (colA), durancin Q (duqQ), enterocin 96 (ent96), enterocin C (entC), enterocin SE-K4 (entSE-K4), enterocins W α and β (enwAB), enterocin Q (entqA), and mundticin KS (munA) were not detected among our isolated strains. Sequenced amplicons were aligned using the MEGA 5.2 software and a maximum likelihood tree was created and presented in **Figure 9**.

Table 2. Bacteriocin structural gene primer sequences used in this study.

Primer	Target Gene	Sequence (5'->3')	Product Size (bp)	Included Genes	Homologous Genes
1	**Avicin A (avcA)** - FJ851402.1		236	avicin A precurson (avcA) immunity protein (avcI) divergicin-like bact (avcB)	sakacin X (sakX)
	Forward	ACG CGA AAT GAA GAA TGT TG			
	Reverse	TTT CAT TTC CGC CAG AAA AC			
2	**Columbicin A (colA)** - EF033111.1		299	columbicin A (colA) hypothetical protein (orfB)	bovicin A (bovA) enterocin as-48
	Forward	TTT TTC TTG GGT TAT TTA CAG GAA			
	Reverse	ATG TGC AAT GGG CAA AAA CT			
3	**Durancin Q (duqQ)** - AB284369.1		384	immunity protein (duqI) durancin Q (duqQ) inducing peptide (duqF)	durancin TW-49 (durM)
	Forward	GCA CTG ATT CCG GCA CTA AT			
	Reverse	CGT AAC TCT AAT GGC GGG AAG			
4	**Enterocin 96 (ent96)** - FJ769024.1		291	enterocin 96	-
	Forward	GTG GAG AGG ACG AAA GGA GA			
	Reverse	TTG ATT AGT GGA GAG GAC GGT TA			
5	**Enterocin mr10A/mr10B (mr10AB)**		247	enterocin mr10A (mr10A) enterocin mr10B (mr10B)	enterocin JSB (entJSB) enterocin NA (entNA) enterocin NB (entNB) enterocin L50A (entL50A) enterocin L50B (entL50B) enterocin 62-6A (ent626A) enterocin 62-6B (ent626B) enterocin RJ-11
	Forward	ATG GGA GCA ATC GCA AAA T			
	Reverse	CAT CCT TGT CCG ATA AAC TGC			
6	**Enterocin C (entC)** - FU862242.1		506	enterocin C1 (entC1) enterocin C2 (entC2) enterocin C immunity (entCI)	enterocin 1081A enterocin 1071B
	Forward	AGG TCC AGC TGC TTA TTG GA			
	Reverse	CCA TTA GAA TGA ATA CGC TAA AGA AA			
7	**Enterocin SE-K4 (entSE-K4)** - AB092692.1		608	enterocin SE-K4 (entSE-K4) enterocin precursor (orf7) entSE-K4 homologue (orf8) entSE-K4 immunity (orf9)	bacteriocin II (D78257.1) enterocin TW-21 Bacteriocin 31
	Forward	ATG TAG AAG CCG CCA CGT AT			
	Reverse	AAT CCC AAT CAT CCC ACA AA			

8	**Enterocin EJ97(ej97a)** - AJ490170.1		104	enterocin ej97	-
	Forward	AAA GCG ATG ATT AAG AAG TTT CC			
	Reverse	TCC CAA GGA TAA CGA CCG TA			
9	**Enterocin Wα/Wβ (enwAB)** - AB600897.1		423	enterocin W alpha (enwA) enterocin W beta (enw B)	-
	Forward	GGG GTT GAA TTA TTG TAG AAA GGA			
	Reverse	AAC TAG CCT CTA CCG CCA CA			
10	**Enterocin Q (entqA)** - DQ832184.1		231	enterocin Q (entqA)	-
	Forward	ATC ACA AAG TGA GCC CCT GT			
	Reverse	TGG TAT CGC AAA ATG GAT GA			
11	**Enterocin P (entP)** - AF005726.1		431	enterocin P (entP) enterocin P immunity (entQ)	-
	Forward	TTC CCC GAA GAA TAC AAA TGA			
	Reverse	AAT TTC TGG GGT GGC TAA TG			
12	**Enterocin A (entA)** - AF240561.1		362	enterocin A (entA) immunity protein (entI)	-
	Forward	AAA ATA AAT GTA CGG TCG ATT GG			
	Reverse	CCA GCA GTT CTT CCA ATT TCA			
13	**Enterocin B (entB)** - U87997,1		257	enterocin B (entB)	enterocin CRL35
	Forward	CAG AGT TCC CAA CTG TTT GCT			
	Reverse	AGC CCA TGC TAG TGG TCCT T			
14	**Enterocin Xα/Xβ (enxAB)** - AB430879.1		321	enterocin X alpha (enxA) enteorcin X beta (enxB)	-
	Forward	GGACAATTTATGGGTAAACAAGC			
	Reverse	TACGTCCACCATTCCAACCT			
15	**Bacteriocin T8 (bacA)** - AB178871.1		469	bacteriocin precursor (bacA) hypothetical immunity protein (bacB)	hiracin JM79 Bac43
	Forward	TTGTCTAGCTGGCATCGGTA			
	Reverse	CCAATAGAAGCCCATCCTCT			
16	**Mundticin KS (munA)** - KC291253.1		285	mundticin KS (munA)	mundticin L (munL) enterocin HF
	Forward	AAA AGG GTG CAG TGT TGA TTG			
	Reverse	TCC ACT GAA ATC CAT GAA TGA			

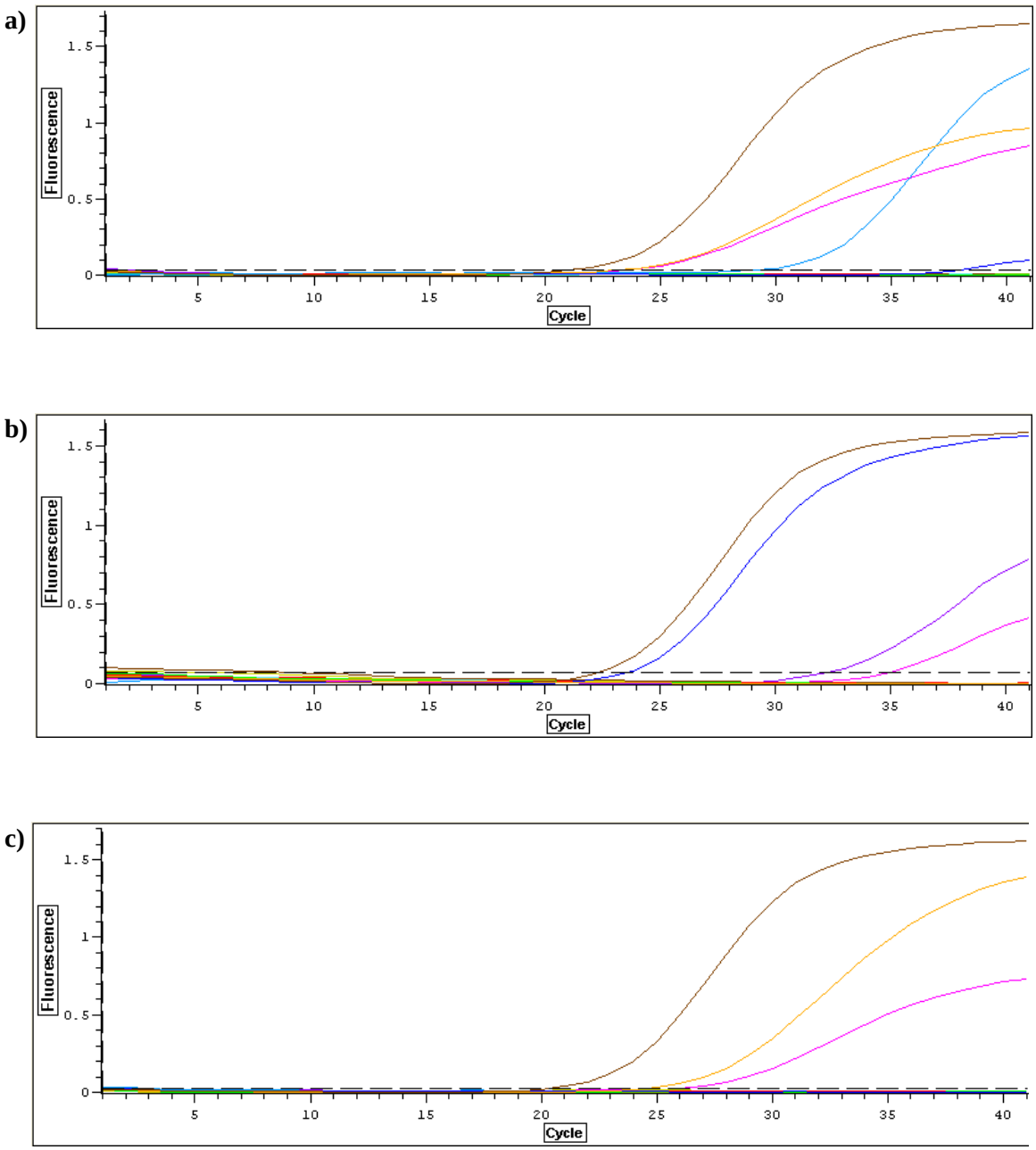

Figure 5. Quantitative PCR amplification plots for *Enterococcus* structural genes.

a) 323RL1 (*Enterococcus hirae*). From high to low fluorescence end points: 16S Control (Ct=22.2), entA (Ct=30.3), munA (Ct=24.8), mr10AB (Ct=24.9), and bacA (Ct=38.3).

b) Milk5 (*Enterococcus faecium*). From high to low fluorescence end points: 16S Control (Ct=22.4), bacA (Ct=23.5), entP (Ct=32.3), and mr10AB (Ct=34.9).

c) FS707 (*Enterococcus durans*). From high to low fluorescence end points: 16S Control (Ct=20.2), munA (Ct=24.3), and mr10AB (Ct=25.5).

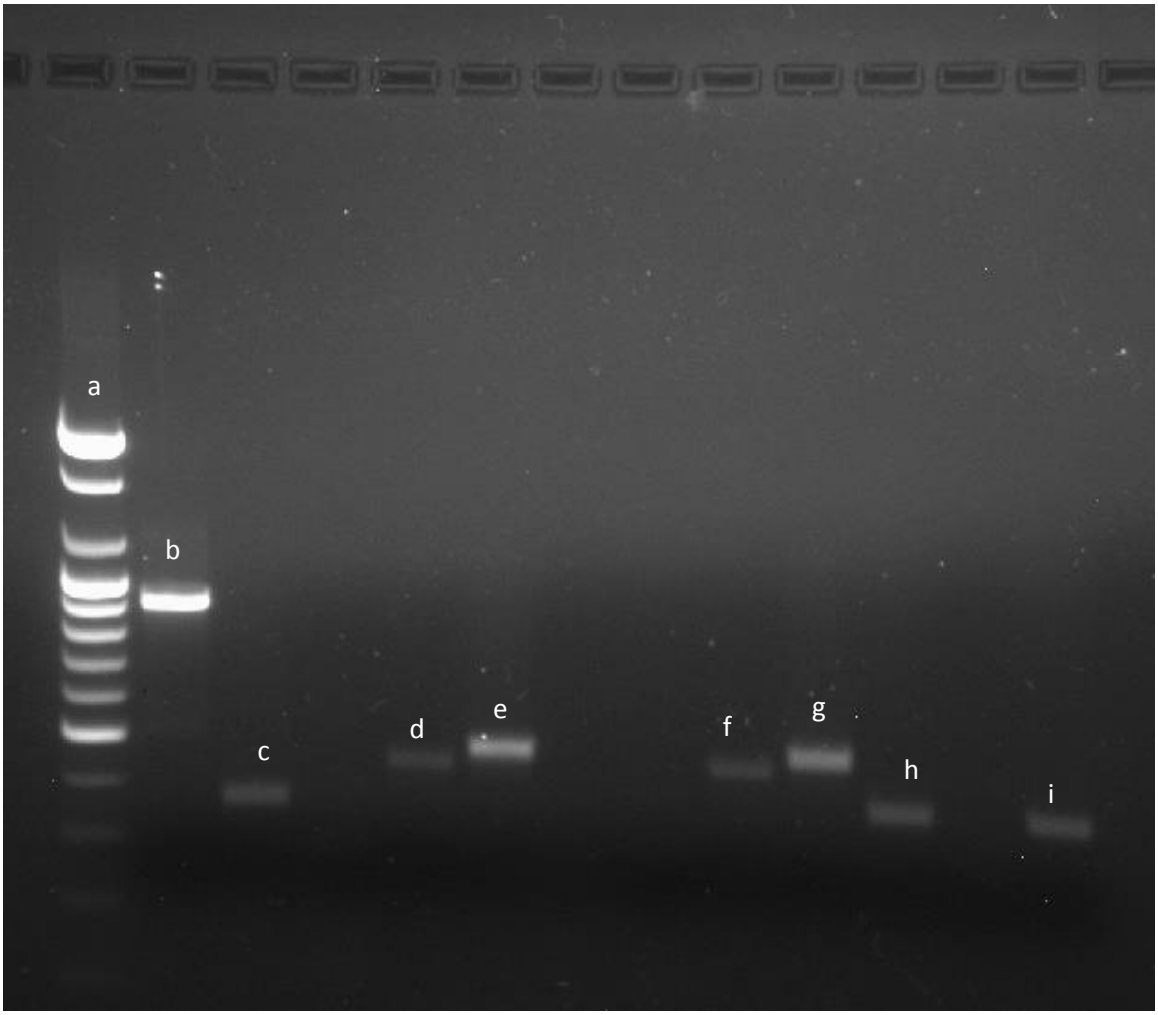

Figure 6. Gel electrophoresis of amplified *Enterococcus* bacteriocin genes.

Wells, from left to right: 100kb Ladder (a), 16S Control (b), NP-7 entA (c), JCP-9 mr10AB, JCP-9 entP (d), JCP-9 bacA (e), JCP B-5 ent96, JCP B-5 mr10AB, JCP B-5 entP (f), JCP B-5 bacA (g), THYME2 entA (h), THYME2 entB, and THYME3 entA (i).

Table 3. *Enterococcus* strains used in this study and sequence homology to the bacteriocin structural gene

Isolate	Species	entA	mr10AB[1]	enxAB	bacA	entP	entB	munA
FS707	*durans*							99%
BJ-12	*faecalis*	100%[2]						
BJ-13	*faecalis*				99%			
BJ-19	*faecalis*	100%						
BJ-27	*faecalis*	100%	96%					
326F	*faecium*	100%	92%	99%	99%			
FS56-1	*faecium*	100%	97%					
FS97-2	*faecium*	100%	99%	100%				
JCP B-5	*faecium*		97%		100%	99%		
JCP M-2	*faecium*		95%		100%	99%		
JCP-9	*faecium*		98%		100%	99%		
Milk12	*faecium*	100%	99%		100%			
Milk5	*faecium*	100%	99%	100%	99%			
NP-7	*faecium*	100%						
Poop4	*faecium*	100%	96%	100%			99%	
THYME2	*faecium*	100%					100%	
THYME3	*faecium*	100%						
323F	*hirae*	100%	97%					
323RL1	*hirae*	100%	96%					99%
341FA	*hirae*	100%	93%			99%		
FS92	*thailandicus*	100%	97%					
RP-1	*thailandicus*	100%						

[1] Homology values in this column are related to the genes for L50A and L50B. L50A and B sequence obtained from NCBI's database.

[2] Homology percentages based on highest "Max Score" by NCBI's nucleotide BLAST program.

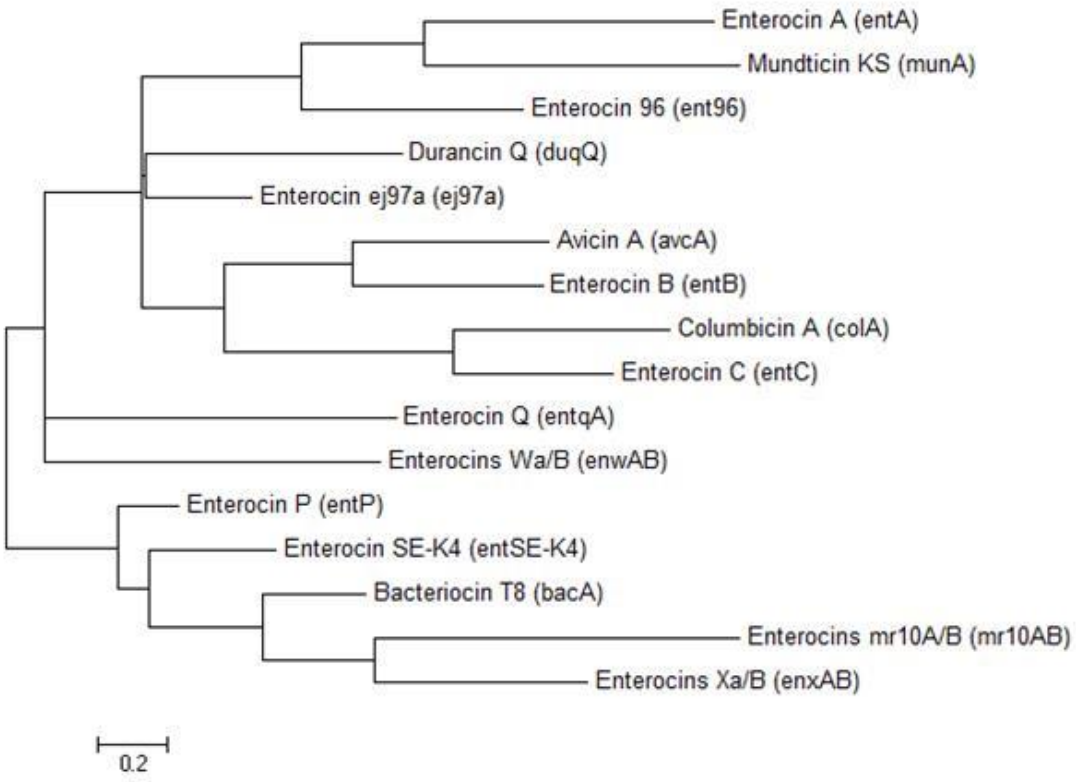

Figure 7. Maximum likelihood homology tree of structural gene sequences used in this study obtained from the Bactibase database.

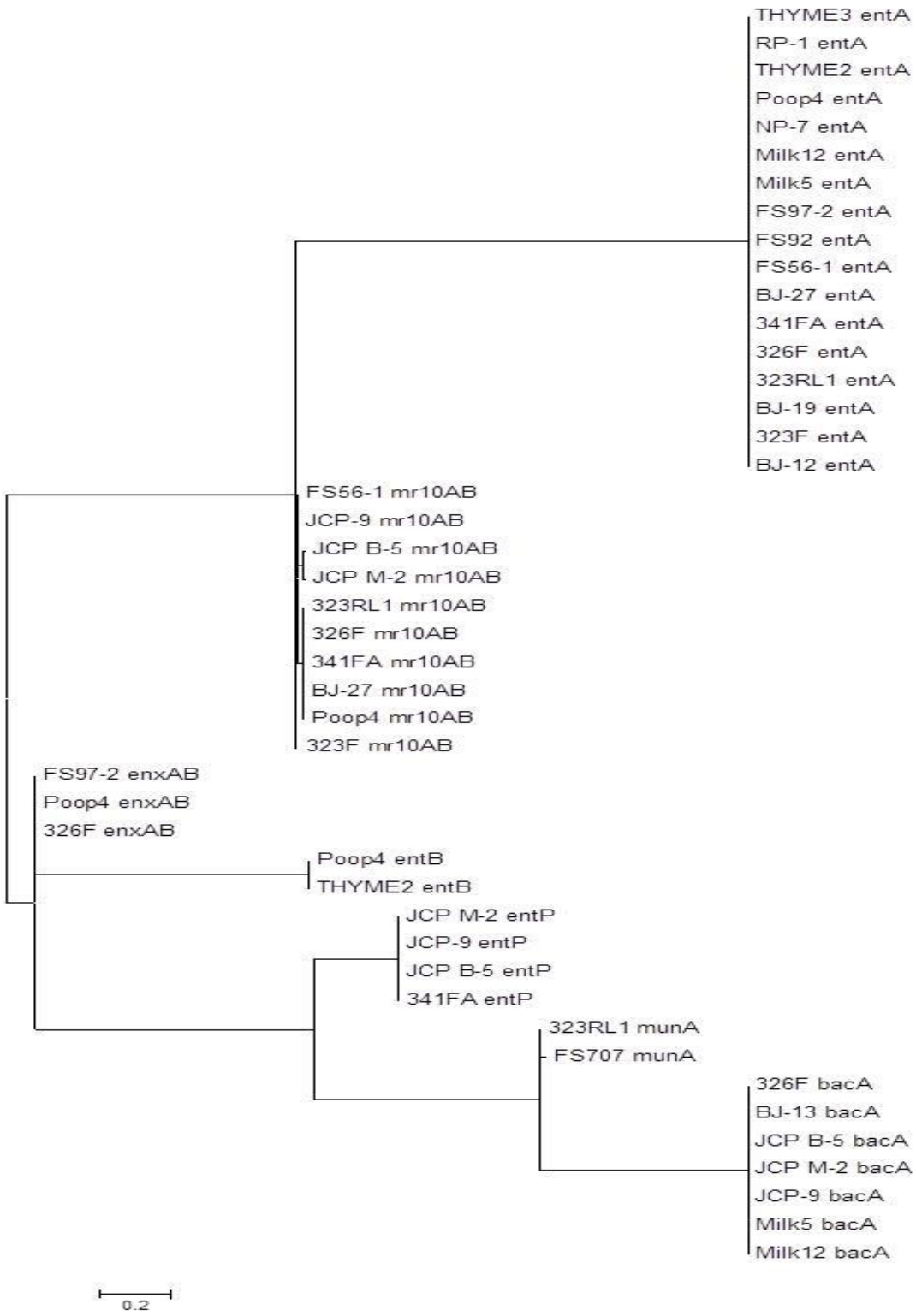

Figure 8. Maximum likelihood homology tree of sequenced structural genes amplified from *Enterococcus* strains isolated in this study

Discussion

Enterococcus spp. are widely distributed within most ecological niches. Of particular interest is their role in the creation of fermented foods and the synthesis of antimicrobial compounds such as bacteriocins. Enterococci are part of the natural flora of the mammalian gastrointestinal tract but can also be the cause of nosocomial infections (Rehaiem et al., 2014). Their presence of part of the flora isolated from artisanal food products made from raw ingredients has been the subject of debate on whether they should be considered as food starter cultures (Kornacki, 2012). Surely, some strains produce virulence factors and some enterococci are listed as BSL-2 strains. However, their ability to produce bacteriocins makes them viable candidates for food biopreservation.

Naming conventions for bacteriocin structural genes are often inconsistent. Of the 16 target structural genes in this study, nine had at least one additional structural gene to which they were highly homologous or identical, with four of them having at least two. For just the structural genes mr10A and mr10B (transcribed together), genes with high homology include enterocin JSB, enterocins NA and NB, enterocins L50A and L50B, enterocins 62-6A and 62-6B, and enterocin RJ-11. Common parts of the bacteriocin gene clusters were not included into the primer design such as ABC transporters and some N-terminal consensus sequences like those found in class IIa bacteriocins. Immunity genes, which typically are preceded by their corresponding structural gene (Aymerich et al., 1996), were included in our primer design. The inclusion of the immunity genes in primer design may interfere with the maximum likelihood tree in the homology comparison of the sequenced bacteriocin genes.

Previous studies indicate that bacteriocin-producing members of *Enterococcus* have no preferential niche in the ecosystem (Nes et al., 2006). Our isolates alone represent a wide distribution of food and environmental samples samples (**Table 1**) such as fecal matter (323F,

326F, 341FA, Poop4), meat (JCP-9/B-5/M-2), dairy (Milk 5/12), rumen fluid (323RL1) and herbs (THYME 2/3). In addition, isolates from the same type of sample displayed similar bacteriocin structural genes.

Enterocin A was the most commonly found bacteriocin structural gene from the 22 isolates we tested in this study. Enterocin A shows sequence homology to class IIa bacteriocins such as the pediocin-like bacteriocins (Aymerich et al., 1996). Given that the isolates are all able to inhibit *L. monocytogenes*, a high frequency of pediocin-like bacteriocins is normal due to their common ability to inhibit *L. monocytogenes* (Cleveland et al., 2001; Wieckowicz et al., 2011). Further analysis to eliminate those that may be potentially pathogenic to humans may allow the remaining strains, or their derived culture supernatant fractions, to be used as biopreservatives in food applications.

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VITA

Christopher David Henning

Candidate for the Degree of

Master of Science

Thesis: ISOLATION AND PHYLOGENETIC ANALYSIS OF BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA FROM RETAIL FOODS

Major Field: Food Science

Biographical:

Education:

Completed the requirements for the Master of Science in Food Science at Oklahoma State University, Stillwater, Oklahoma in December 2014.

Completed the requirements for the Bachelor of Science in Microbiology/Cell and Molecular Biology at Oklahoma State University, Stillwater, Oklahoma in 2011.

Experience:

Research Assistant, Physiological Sciences Laboratory, Stillwater, OK (*April 2011 – December 2011*) – Dr. Guangping Chen

- Extract and purify RNA and protein, western blotting, RT-qPCR, radioactive enzyme assays, laboratory animal handling

Research Assistant, Microbial Ecology Laboratory, Stillwater, OK *(August 2011 – December 2011)* – Dr. Babu Fathepure

- Isolate acid-producing bacteria, PCR, gel electrophoresis, gas chromatography, molecular cloning

Graduate Research Associate, Food Microbiology Laboratory, Stillwater OK (January 2012 – July 2014) – Dr. Peter Muriana

- qPCR, *Listeria*/*E. coli* swabbing, teach Food Microbiology class, DNA extraction and purification, microscopy