NOVEL ANTIMICROBIAL ACTIVITY OF LACTIC ACID BACTERIA ISOLATED FROM TARO PEELS

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ABSTRACT

Pathogenic bacteria have caused a large number of foodborne illnesses and great economic loss in food and animal production. *Salmonella* and *Listeria* are two notorious foodborne pathogens with high prevalence and severity. It is urgently needed to find effective measures to control these pathogens. Taro is the staple of native Hawaiian diet and an important part of Polynesian culture. Taro peels, a by-product of poi, are removed from taro after pressure cooking. Pigs were found to recover from gastrointestinal illness when fed pressure-cooked taro peels. It was hypothesized that lactic acid bacteria (LAB) survived the high-pressure condition may be present in taro peels and responsible for the recovery.

The first objective of this study was to identify dominant bacterial species present in taro peels and determine their antimicrobial potential against pathogenic bacteria. Samples from four poi manufacturers on Oahu and Maui were collected and analyzed. After 48 h of natural fermentation, LAB isolates were obtained on selective MRS agar and identified by RAPD-PCR and 16S rDNA gene sequencing. Representative LAB strains were tested for their acid and bile tolerance. Culture supernatants of LAB isolates with strong acid and bile tolerance were evaluated on their bactericidal properties against *Salmonella Typhimurium* and *Listeria monocytogenes*. A total of 159 isolates belonging to 11 different LAB species were obtained, of which majority were *Leuconostoc* spp. *L. mesenteroides* strains accounted for approximately 40% of all isolates; *L. lactis*, *L. pseudomesenteroides*, *Lactococcus lactis*, and *Weissella confusa* also frequently occurred in the sample. Four isolates #67, #97, #101 and #137 exhibited high tolerance to low pH and bile, thereby enabling them to survive in the gastrointestinal environment. The LAB isolates showed different antimicrobial effects on the tested pathogens. *S. Typhimurium* and *L. monocytogenes* died in the culture supernatants within 2 h and 8 h, respectively.

Moreover, this study also aimed at exploring the production of bacteriocins by the LAB isolates. Agar well diffusion assay was performed to determine the sensitivity of antimicrobials in the culture supernatants to proteinase K. Additionally, the inhibitory effect of neutralized supernatants on the indicator bacteria was evaluated by spot-on-lawn and broth enumeration assays. Proteinase K treatment reduced the inhibition zone of certain
supernatants against *L. monocytogenes*. Neutralization eliminated the antimicrobial effect of organic acids toward the tested pathogens. Only the supernatant of one isolate #137 *Lactococcus lactis* still retained the bactericidal activity against *L. monocytogenes*. PCR-based methods were used to detect the bacteriocin-encoding genes in the LAB isolates. No gene related to class IIa bacteriocins has been amplified, while two bacteriocin structural genes were amplified by primers NisZ and Bac147 using genomic DNA of isolate #137 as template. This is the first *Lactococcus lactis* strain reported to carry these two bacteriocin genes simultaneously. The anti-*Listeria* bacteriocins produced by the *Lactococcus lactis* strain were heat-stable but sensitive to proteinase K and pronase E. Isolate #137 is potentially a novel bacteriocin-producing *Lactococcus lactis* strain.

Both organic acids and bacteriocins may account for the antimicrobial activity of LAB isolated from taro skins. These isolates could potentially be used as probiotics to fight bacterial infections and confer other health benefits in humans and animals. With the ability to survive pressure-cooking, these isolates have potential to be used in food production. Bacteriocins produced by the isolates would offer a very promising biocontrol tool for inhibiting pathogenic bacteria in food.
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CHAPTER 1. INTRODUCTION

Pathogens causing foodborne illness are of great concern to the public. Every year, approximately 1.03 million cases of salmonellosis and 1,600 cases of listeriosis occur in the United States (Scallan et al., 2011). Human illnesses caused by *Salmonella* and *Listeria monocytogenes* cost 2.3 and 2.9 billion dollars annually, respectively (ERS-USDA, 2004). These pathogens can infect farm animals, such as cattle, sheep, poultry and pigs, and eventually spread to various foods. Furthermore, the infections might cause gastroenteritis, abortion, and even death in the animals. Animal health certainly plays an important role in ensuring the safety of food supply. Since antibiotic resistance in humans and animals has emerged as one of the preeminent health concerns worldwide, there is an urgent need to find alternative measures to fight bacterial infections.

Lactic acid bacteria (LAB) might offer a viable option to controlling pathogenic bacteria *in vitro* and *in vivo*. LAB display considerable antimicrobial activities by producing organic acids, hydrogen peroxide, and/or low-molecular-mass peptides (bacteriocins) (Makras et al., 2006). Certain LAB strains have been recognized as a main source of probiotics, which are defined as “live microorganisms [that] when administered in adequate amounts confer a health benefit on the host” (USDA). In addition to combating pathogens, LAB may have other positive effects, including balancing intestinal microbiota, lowering fecal enzyme activity, treating lactose intolerance, immune enhancement, and reducing the reoccurrence of bladder cancer (Salminen and Gueimonde, 2004). Fermented dairy products, such as yogurt and buttermilk, have commonly been used to deliver probiotics. A number of LAB strains with probiotic potential have been isolate from several naturally-fermented, non-dairy foods including kefir, miso, natto, and tempeh (Santosa et al., 2006).

Taro is a very popular crop in many tropical areas. Majority of processed taro in Hawaii is made into poi, a traditional staple food for Native Hawaiians (Allen and Allen, 1933; Greenwell, 1947). Poi is a paste derived from pounded, pressure cooked taro corms. During storage at room temperature, fresh poi develops a strong, sour flavor. The souring process is a natural fermentation proceeding with LAB and yeast that may originate from
the corms and the processing environment. These microorganisms might survive pressure-cooking and be more likely to be developed as protective cultures in food products. Previous research indicated the presence of three *Lactobacillus* species and two *Streptococcus* species in cooked peeled taro (Allen and Allen, 1933). Huang et al. (1994) identified *Lactococcus lactis* as the most prevalent bacteria in sour poi, representing about 85% of the microflora. It was reported that poi could support the growth of bacteriocin-producing bacteria (Muller et al. 2002). Yet the mechanism by which LAB present in poi inhibit pathogenic bacteria is still unknown.

In a local swine farm, it was observed that piglets suffering gastrointestinal illness from post-weaning stress could recover quicker when they were fed fermented taro skins. Thus, as a by-product of poi manufacture, the taro skins were assumed to contain LAB and possess antimicrobial potential as poi does. To verify the assumption, taro skin samples were collected from four poi manufacturers on Oahu and Maui, followed by microbial analysis and antimicrobial evaluation. The specific objectives of this research were to:

1. Identify dominant LAB species in fermented taro skins collected from different poi manufacturers;
2. Test the tolerance of selected LAB isolates under low pH and high bile conditions;
3. Evaluate the antimicrobial activity of promising LAB isolates against *S. Typhimurium* and *L. monocytogenes*;
4. Determine the production of possible bacteriocin-like compounds and their contribution to the antimicrobial potential of promising LAB isolates.
CHAPTER 2. LITERATURE REVIEW

2.1 Foodborne pathogenic bacteria

Foodborne diseases are caused by ingestion of foodstuffs contaminated by pathogenic microorganisms or toxic chemicals (WHO). They are a growing public health problem occurring worldwide. Gastrointestinal illnesses, such as diarrhea, vomiting, nausea and abdominal pains, are the most common symptoms of foodborne diseases. They could also result in life-threatening complications with neurological, gynaecological, or immunological symptoms. It is estimated that foodborne pathogenic bacteria cause 3.6 million illnesses annually in the United States, consisting of 1,093 deaths and 50,673 hospitalizations (Scallan et al., 2011). The contamination of food by pathogenic bacteria is a big issue in food safety. Among known pathogenic bacteria, *Salmonella* and *Listeria* are two common and typical pathogens involved in foodborne illnesses. According to CDC, *Salmonella* is estimated to cause one million illnesses annually in the United States, with 19,000 hospitalizations and 380 deaths. *Salmonella* is the leading cause of foodborne diseases. *Listeria*, another deadly foodborne pathogen, particularly threatens pregnant women, older adults, and people with weakened immune systems. It is the third leading cause of death from food poisoning.

2.1.1 *Salmonella*

*Salmonella* spp. are Gram-negative, rod shaped bacteria that can cause salmonellosis in not only humans but also animals. More than 2000 different serotypes of *Salmonella* have been identified, among which *S. Enteritidis* and *S. Typhimurium* are the most significant foodborne pathogenic serotypes (Lax et al., 1995). The symptoms of *Salmonella* infection include gastroenteritis and typhoid fever (Cummings et al., 2012; Forshell and Wierup, 2006). Even though the pathogens might contaminate food products during processing or handling, considerable amount of them originates from infected farm animals, such as cattle, swine and
chicken (Forshell and Wierup, 2006). In addition to human health concern, salmonellosis causes animal producers huge economic loss due to poor production efficiency and even death of infected animals. It has been estimated that diseased swine affected by *Salmonella* Choleraesuis leads to $100 million loss each year for pork producers in the United States (Schwartz & Ritter, 2002).

### 2.1.2 Listeria monocytogenes

*Listeria* spp. are Gram-positive, rod-shaped and facultative anaerobic bacteria widely distributed in the environment. Among the ten species of *Listeria*, *L. monocytogenes* is the most important foodborne pathogen accounting for listeriosis in humans and animals as well. Human cases of listeriosis are almost exclusively caused by *L. monocytogenes*. Non-perinatal infection of this pathogen causes septicemia, meningitis, and encephalitis. In pregnant women, *L. monocytogenes* might cause stillbirth or abortion of the fetus. CDC received 831 reports of foodborne listeriosis in 2012, and it is estimated that 1600 listeriosis cases including 260 deaths occur annually in the United States.

Infection of animals by *Listeria* potentially leads to abortion, encephalitis and septicemia, resulting in great loss in animal industry. *L. monocytogenes* causes majority of listeriosis in animal, which has been associated with abortion of many species of animals, especially ruminants such as bovine and sheep (Gray et al., 1956; Hathaway, 1997; Low and Donachie, 1997; Stockton et al., 1954; Young and Firehammer, 1958). Though relatively less frequently, another species of *Listeria*, *Listeria ivanovii* has also been implicated in abortion of cattle and sheep, and septicemia in sheep (Alexander et al., 1992; Chand & Sadana, 1999; Laureyns et al., 2008).

High resistance of *Listeria* to a wide range of environmental stresses, such as refrigeration temperature, low pH down to 4.4, and high salt concentration, has been well known (Convey et al., 2000; Dimitrijevic et al., 2006; Ivy et al., 2012;). Moreover, previous exposure to mild acid environment improves the acid tolerance of *L. monocytogenes* (Gahan et al., 1996; Kroll & Patchett, 1992). Although sufficient heat is able to inactivate *L.*
monocytogenes, certain foods without heat treatment before consumption including fresh produce and ready-to-eat foods, and animal feed have high possibility of transmitting the pathogen. Therefore, it is a great challenge for both food producers and animal raisers to protect their products from L. monocytogenes.

2.2 Biocontrol of foodborne pathogens by LAB

Since pathogenic bacteria pose a serious threat to human and animal health, it is imperative to find effective and economical strategies to control them. In the food industry, thermal treatment, chemical sanitizing, and addition of food preservatives are common methods for disinfection of pathogens (Dubal et al., 2004; King et al., 2005; Spricigo et al., 2013). However, these traditional approaches have their own disadvantages, such as inadequate efficiency, decreasing the food quality, and limited application for fresh produce or ready to eat foods. In animal production, current measures including hygienic control on the farm and vaccination are not satisfied (Wong et al., 2014). Feeding antibiotics is one of the most effective approaches to protect food animals from gastrointestinal disease and other illness caused by foodborne pathogens. Nevertheless, an increasing concern about transmission of antibiotic resistance to humans has led to dramatic decline in the use of antibiotics. The anti announced the prohibition of using antibiotics as growth promoters in food animals in 1999 (Graham et al., 2007). As a result, there is a growing interest in biocontrol, which utilizes naturally occurring microorganisms or biological agents produced by them to suppress or even eliminate pathogens.

The application of LAB in biocontrol is promising since they are generally recognized as safe (GRAS) and naturally present in fermented foods with long history and without any safety risk. LAB possess antimicrobial activity against foodborne pathogens mainly due to competition for nutrients and production of bactericidal metabolites (Holzapfel et al, 1995). A number of LAB species have been proven to be effective protective culture in a wide range of food products. Pediococcus acidilactici and Lactobacillus sakei have been studied in bio-preservation of fermented sausage, fresh or ready-to-eat meat, and other meat products.
sicne more than 20 years ago (Albano et al., 2009; Amézquita and Brashears, 2002; Castellano et al., 2012; Coventry et al., 1995; Schillinger et al., 1991; Vermeiren et al., 2006). To date, fermentation starters, such as SafePro® (CHR Hansen, DK) and Bovamine Meat CultureTM (NPC, US), are commercially available for *L. monocytogenes* control in the meat industry (Ghanbari, 2013). Several *Carnobacterium* strains showed inhibitive effect on *L. monocytogenes* in cold smoked salmon with little influence on the food quality (Brillet et al., 2005; Nilsson and Huss, 1999; Yamazaki et al, 2003). The utilization of LAB strains as protective culture also benefits fresh or minimally processed vegetables and fruits by suppressing the growth of foodborne pathogens (Scolari and Vescovo, 2004; Torriani et al., 1997; Trias et al., 2008).

Moreover, LAB and their metabolites have been used in animal production. *Enterococcus*, *Streptococcus*, *Pediococcus*, *Bifidobacterium* and *Lactobacillus* are main LAB genera associated with reduction of colonization of pathogenic bacteria in poultry and livestock. Prevention of *S. Enteritidis* colonization in chicken has been achieved by oral gavage of *Lactobacillus salivarius* (Pascual et al., 1999). Feeding lambs with *Streptococcus faecium* resulted in reduced fecal shedding of *Escherichia coli* O157:H7 in the animal (Lema et al., 2001). Compared to the use of a single strain, there have been more studies dealing with utilization of mixed culture of different LAB strains and even species, which showed higher efficiency (Arguello et al., 2012). It has been demonstrated that a mixed culture of two strains of *Lactobacillus murinus* and one strain each of *Lactobacillus salivarius* subsp. *salivarius*, *Lactobacillus pentosus*, and *Pediococcus pentosaceous* significantly improve the clinical and microbiological outcome of *Salmonella* infection in pigs (Casey et al., 2007). In the same study mentioned previously, a higher reduction of *S. Typhimurium* was achieved by a mixture of *S. faecium, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus fermentum* and *Lactobacillus plantarum* than *S. faecium* treatment only (Lema et al., 2001).
2.3 Probiotics

2.3.1 History of probiotics

The modern concept of probiotics was first introduced by Elie Metchnikoff based on the study of a Bulgarian microbiologist, who disclosed the health benefits of Bulgarian yogurt and identified *Lactobacillus delbrueckii* ssp. *bulgaricus* as a functional microorganism in the yogurt (Hume, 2011). Though the concept initiated from Europe, more studies associated with probiotics have been done in North America after Metchnikoff’s death in 1916. Researchers in the United States proposed that *L. acidophilus*, an organism isolated from the gut, potentially provides more benefits than *Lactobacillus delbrueckii* ssp. *bulgaricus* from yogurt (Azizpour et al., 2009). Their assumption was supported by a number of trials regarding constipation treatment by *L. acidophilus* feeding. In the late 1940’s, more research been conducted to reveal the microbial composition in the gut, followed by isolating more species of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* from the gut. As a result, new LAB species other than *L. acidophilus* have been studied for probiotic potential from then on (Ruller, 1995).

According to Fuller (1992), Lilley and Stillwell first used the word probiotic to describe “substances secreted by one microorganism which stimulated the growth of another” in 1965. A few years later, a further definition was given by Parker (1974) as "organisms and substances which contribute to intestinal microbial balance" (Hume, 2011). However, many substances included antibiotics can be considered as probiotic according to this definition. Thus, Parker’s description is too broad to be accepted by many researchers (Azizpour et al., 2009). More recently, Fuller (1992) defined probiotics as “mono- or mixed cultures of living microorganisms which beneficially affect the host by improving the properties of the indigenous microbiota” by avoiding the controversial word “substances” and emphasizing the viability of probiotics. Currently probiotics are officially defined as “live microorganisms that when ingested in adequate amounts, are expected to confer health benefits to the host”
To date, numerous LAB strains have been discovered in different food products and evaluated for their probiotic potential. Lactobacilli from a Greek dry-fermented sausage were identified and their probiotic properties were studied (Papamanoli et al., 2003). *Enterococcus faecium* strains isolated from cow milk have also been reported to exhibit good probiotic activities and have high potential to produce functional fermented dairy products (Banwo et al., 2013). Peres et al. (2014) isolated *Lactobacillus* strains from fermented Portuguese olive as potential candidates for a probiotic culture. Blana and coworkers (2014) successfully applied the LAB strains obtained from industrially fermented olives as starter cultures to green olive fermentation with high performance.

### 2.3.2 Characteristics of probiotics

Several characteristics and criteria should be taken into account for the evaluation of probiotic potential. Gordon et al. (1957) proposed the requirements for selecting lactobacilli for therapy as followed: non-pathogenic, normal inhabitant of intestine, capability of gut colonization. General speaking, the criteria for selection of probiotics can be divided into four aspects: safety criteria, technological criteria, functional criteria, and desirable physiological criteria (Azizpour et al., 2009).

Safety criteria include the origin, pathogenicity, and intrinsic properties of potential strains. First of all, a probiotic is supposed to be non-pathogenic and non-toxic, causing no harmful effect to the host. The origin of the strain is significance for the selection of probiotics. For instance, LAB strains isolated from fermented food with a long history of safe use in the food industry are good candidates of probiotics. Research also indicates that antibiotic resistance of probiotics is another important safety criterion due to the potential transfer of antibiotic resistant genes to the host (Azizpour et al., 2009). For the technological criteria, the genetic stability, sensory properties, commercial productivity and viability during processing and storage are the main features should be considered.

The ability of strains to colonize the gut and attach the gut epithelial lining is the basis
of the determination of probiotic. Although a variety of LAB strains show antimicrobial activity, the effect cannot be performed if the strains are not able to metabolize in the gastrointestinal system. Therefore, in vitro assessment of tolerance to low pH and high bile environment, which simulate the inhibitory condition in the gut, is necessary for functional evaluation of probiotics. Furthermore, adhesion of strains to the intestinal surface, which allows them to persist in the flow of digesta, is another required property. Using pieces of intestinal tissues, cell lines or mucus to assess the adhesion in vitro has been demonstrated in previous studies (Morelli, 2007). Even though in vitro study provides preliminary data, strains showing high adhesion in vitro not necessarily perform well in the host. Thus, in vivo study for the functional criteria is highly recommended.

A wide range of physiological benefits associated with probiotics has been disclosed, including antimicrobial effect against gastrointestinal pathogens, immunomodulation, reduction of risk of carcinogenicity, alleviation of lactose intolerance, treatment of allergy, benefits to hypercholesterolemia and cardiovascular system (Azizpour et al., 2009). Different probiotic strains are related to one or several of above benefits, among which enhancing gastrointestinal health is the most significant effect of most probiotic strains.

2.3.3 Role of probiotics in gastrointestinal health

A variety species of LAB possess the probiotic potential due to their positive effect to the gastrointestinal microbiota. Their colonization in the gut antagonizes the pathogenic bacteria causing disorder of gastrointestinal tract, and meanwhile improves the growth of beneficial bacteria, thereby modulating the gut microbiota (Correia et al., 2012). In another word, probiotics play an important role to the positive balance of microbial community in the gut, attributed to various mechanisms.

First of all, the metabolites produced by probiotics result in a physiologically restrictive environment preventing the colonization of pathogenic bacteria. Bifidobacterium breve has been reported to reduce the luminal pH by producing acetic acid, thereby increasing the survival of animals infected by E. coli O157:H7 (Asahara et al., 2004).
Additionally, probiotics compete with pathogenic bacteria for nutrients or substrates required for their metabolism.

Colonization of enteric pathogens depends on their adherence to the epithelial lining of the gut as well. Previous research proved that probiotics could enhance secretion of colonic mucin in vivo and in vitro, protecting the epithelial cells from attachment by pathogens. The capacity of probiotics to binding pathogenic bacteria and occupying receptor-binding sites on the epithelial lining also significantly inhibits the adhesion of pathogens to the gut (Correia et al., 2012). These properties of probiotics not only antagonize harmful organisms but also improve the intestinal barrier function. A mixed culture of different strains of Lactobacillus, Bifidobacterium and Streptococcus has been successfully used to normalize barrier integrity in excised tissue from mice (Madsen et al., 2001). Several Lactobacillus species are related to barrier function enhancement in different models of colitis (Ng et al., 2009).

During the colonization of probiotics in the gut, various antimicrobial agents can be produced, and they are important contributors to the antimicrobial activity of probiotic strains. These antimicrobial compounds include organic acids, ethanol, hydrogen peroxide, bacteriocins, and other low-molecular-mass peptides (Makras et al., 2006). It cannot be denied that organic acids, including lactic, acetic, formic, propionic and butyric acids, are the determining factor accounting for the bactericidal effect of probiotics. Most LAB species are able to oxidize lactate to hydrogen peroxide, which is able to inactivate microorganisms depending on the concentrations applied and environmental factors (Reis et al., 2012). Both ethanol from the heterofermentative pathway and diacetyl from excess pyruvate related to citrate metabolism are common products of numerous LAB strains that conveying antimicrobial effect (Rattanachaikunsopon and Phumkhachorn, 2010).

2.4 Bacteriocins

Bacteriocins produced by numerous species of bacteria are ribosomally synthesized antimicrobial peptides or proteins, which are considered to have relatively narrow spectrum
of bactericidal activity (De Vuyst and Leroy, 2007). Nisin, the most extensively characterized bacteriocin produced by *Lactococcus lactis*, is the only bacteriocin with GRAS status for use in specific foods and currently used as a natural food preservative in many countries. The bacteriocins produced by LAB have been explored over many years, focusing on their classification, inhibitory spectra, mechanisms of action, and applications in food and animal production.

### 2.4.1 Classification of bacteriocins

There are a numbers of studies associated with the classification of bacteriocins. To date, four classes of bacteriocins have been defined according to the classification procedure established by Klaenhammer and Nes (2004), which is dependent on their chemical formula, molecular structure and thermal stability.

Class I is defined as lantibiotics, a small and heat-stable peptide containing lanthionine, which is a post-translationally modified amino acid (Reis, 2012). Nisin is a representative of Class I bacteriocins, targeting not only a broad range of gram-positive pathogenic bacteria but also spores of *Bacillus* and *Clostridium* (Daeschel, 1989).

Class II bacteriocins are small, heat-stable but non-lanthionine-containing peptides, further divided into two sub-groups Class IIa and Class IIb. Class IIa are anti-*Listeria* bacteriocins composed of pediocin-like bacteriocins, while Class IIb are called two-peptide bacteriocins showing the antimicrobial activity only by synergistic work of two separate peptides. Class II bacteriocins do not undergo post-translational modifications as Class I bacteriocins do. Therefore, the structure of Class IIa bacteriocins is relatively simple and they are the most investigated among four classes in terms of production and structure-function relationship (Calo-Mata et al., 2008; Eijsink et al., 2002). In spite of less effectiveness against spores, the wide distribution of Class IIa bacteriocins and their strong antimicrobial activity toward close-related pathogenic bacteria, particularly *L. monocytogenes*, make them a promising bacteriocins to be used in food and animal production.

The last two classes of bacteriocin are less common and require further research on
their characteristics. Class III bacteriocins consist of large (>30 kDa), lytic proteins with less applicable potential due to their heat-instability. Undefined mixture of proteins, lipids and carbohydrates are classified in Class IV in many studies, whereas withdraw of this class has been proposed by some scientists (Cotter et al., 2005; Heng and Tagg, 2006).

2.4.2 Production and inhibitory activity of bacteriocins

Bacteriocins are metabolites produced during the growth of bacteriocin-producing bacteria. Previous research demonstrated that bacteriocin production is a growth-dependent physiological trait following primary metabolite kinetics, highly affected by cultivation conditions such as temperature, pH and nutrient availability (De Vuyst and Leroy, 2007; De Vuyst et al., 1996; Lejeune et al., 1998). Based on the consequence of many studies, the optimal pH and temperature for bacteriocin production are generally lower than those for bacterial growth (De Vuyst et al., 1996; Krier et al., 1998; Parente & Ricciardi, 1994). According to Krier et al. (1998), the optimal temperature was 20°C and 25°C for the production of two bacteriocins by Leuconostoc mesenteroides subsp. mesenteroides FR52. The highest yield of mesenterocin 52A and mesenteroacin 52B were obtained at pH 5.5 and 5.0, respectively. They also proposed that the production of these two bacteriocins was stimulated by slow growth rates of the producers. In another study by De Vuyst et al. (1996), it was claimed that reduction of pH would decrease adsorption of bacteriocin molecules to the producer cells, consequently enhancing the yield of bacteriocins. The influence of nutrients can be various to different species. Appropriate concentration of NaCl has been reported to promote the bacteriocin production by Lactobacillus pentosus B96 and Lactobacillus amylovorus DCE 471 (Delgado et al., 2005; Neysens et al., 2003). However, the presence of NaCl reduced the bacteriocin production by Lactobacillus curvatus strain LTH 1174 (Verluyten et al., 2004).

Class I bacteriocins exhibit their antibacterial activity by interacting with lipid II, which is responsible for transporting peptidoglycan subunits from the cytoplasm to the cell wall. Therefore binding of bacteriocins to lipid II inhibits the cell wall synthesis and causes
the death of cell. Moreover, they are also able to use lipid II as a docking molecule to enable pore formation in the cell membrane. As a result, cellular materials would leak out of the cell and then death occurs (Cleveland et al., 2001; Reis, 2012). Regarding to Class II bacteriocins, their amphiphilic helical structure enables the insertion into cell membrane of the targets, thus depolarizing the membrane and killing the cells (Reis, 2012). Bacteriocins produced by LAB generally inhibit closely related Gram-positive bacteria, such as *L. monocytogenes* and *Staphylococcus aureus* (Vuyst and Vandamme, 1994). There are some bacteriocins showing inhibitory effect on Gram-negative bacteria under certain conditions. But the antimicrobial activity is observable only when the outer membrane of target cells is destroyed by chemical or physical approaches such as acid treatment, osmotic shock, pulsed electric field and high-pressure treatment (De Vuyst and Leroy, 2007; Stevens et al., 1991).

### 2.4.3 Detection of bacteriocin-producing LAB strains

Identification of bacteriocin-producers can be achieved via function-based and molecular-based strategies. Agar diffusion (spot and well) and turbidimetric assays are traditional and commonly used techniques for determination of bacteriocin production. These methods are able to measure the inhibition of growth of sensitive indicator bacteria by determining the size of inhibition zone or quantifying the concentration of target bacteria. Although they have drawbacks such as time consuming and low sensitivity, no requirement of advanced equipment or skill makes them easy to perform and useful in many settings. Several studies suggest that appropriate modification of plating methods allow the improvement of the sensitivity. For instance, lower agar concentration (0.75%) than conventional level (1.5%) enables a 21% increase in sensitivity of agar diffusion assay (Wolf and Gibbons, 1996). Addition of 1% Na$_2$HPO$_4$ into the agar medium potentially avoids the false positive results caused by production of organic acids.

With further genetic characterization of bacteriocin-producing LAB, molecular-based strategies have been developed by numerous researchers. PCR-based detection of class IIa bacteriocin-producers are the most explored since most bacteriocins in this group have a
conserved N-terminal amino acid sequence. Therefore, primers that target the gene sequence corresponding to this conserved region can be designed and used for PCR amplification of the genes coding for the bacteriocins (Wieckowicz et al., 2010). However, bacteriocins in other classes do not have a conserved amino acid sequence like class IIa bacterocins, even though they are in the same group. A bacteriocin PCR array has been developed using 42 pairs of primers designed on the coding strands for 42 known LAB bacteriocin structural genes available in the GenBank. This PCR-based detection designed for as many bacteriocins as possible, targeting various species of Lactobacillus, Pediococcus, Lactococcus and Leuconostoc, regardless of what class of bacteriocins they produce (Macwana et al., 2012). Compared to the conventional functional assay, PCR-based detection offers benefits including high sensitivity, cost-effectiveness, and rapid operation. Other novel analytical methods for detection of bacterocins have been studied by far, such as enzyme-linked immunosorbent assay, bioluminometric assay, flow cytometric assay, and microplate assay (Bouksaim et al., 1998; Chen et al., 2007; Nuding et al., 2006; Virolainen et al., 2008). However, these assays may be time-consuming, irreproducible, or require advanced equipment, thus not commonly used.

2.5 Fermented taro

2.5.1 Poi fermentation

Poi is a traditional staple food for native Hawaiians manufactured by simply grinding or pounding the pressure-cooked taro corms into a paste and mixing with water. Although poi has largely been replaced by grain foods as the staff of life in the diet of most present residents in Hawaii, it is still a popular and important source of carbohydrates for many people. According to US Department of Agriculture (USDA), 370 out of 400 acres of taro in Hawaii were grown for poi manufacture in 2012. There are many varieties of taro in Hawaii, such as Chinese taro, dasheen (Japanese taro), Samoan taro, and Lehua, the most significant variety for poi production (Agricultural industry, 1990). Taro corms contain a large amount of
carbohydrates in the form of starch and small amount of fat and protein. They are also a good source of potassium and fiber.

Fermentable sugars in taro enable the growth of LAB and yeast during the fermentation process, which turns the plain taro paste into sour poi. The fermentative microorganisms may originate from natural sources including the soil taro is grown in, the plant’s surface, or from the processing equipment used to manufacture poi (Huang et al., 1994). During the thermal process, microorganisms originally present in taro corms have to go through high pressure conditions, which are deadly to most organisms. However, due to their high-pressure tolerance and the potential protective effect of taro skins with rough surfaces, certain LAB and yeast might be able to survive the pressure cooking. They are responsible for flavor, aroma, and textural properties in sour poi. Because the natural microbial floral in the raw materials may not always be the same, it is difficult to produce poi with consistent characteristics. Few studies have been conducted on the fermentative microorganisms present in poi. Allen and Allen (1933) determined there were at least three Lactobacillus and two Lactococcus species within poi but did not endeavor to create a complete microbial profile. A more recent study found that Lactococcus lactis is the most prevalent bacteria in poi (Huang et al., 1994). Muller et al. (2002) reported the presence of Leuconostoc, Enterococcus and Streptococcus species in freeze-dried poi. Moreover, Lactobacillus delbruckii, Lactococcus lactis, Leuconostoc citreum, Leuconostoc lactis and Weissella confuse were isolated from fresh poi and Lactobacillus plantarum was isolated from sour poi by He et al. (2003). Furthermore, since wetland taros grow in wet areas which are more favorable to anaerobic microorganisms, the quantity and diversity of LAB are potentially higher than those of LAB in dryland taros.

2.5.2 Antimicrobial potential of fermented taro skins

There are two ways to remove the skins from taro in poi manufacture, either peeling before or after cooking. Peeling after cooking is more common in large-scale production. The skins peeled post-cooking still contain certain amount of taro flesh, which offers plenty of
nutrients for microorganisms to consume. In a local pig farm, it was observed that piglets after weaning had high incidence of diarrhea. Feeding the piglets with taro peels helped them recover from diarrhea. Given the occurrence of various LAB species in poi, it was hypothesized that the processed taro peels might have undergone fermentation and contained probiotic bacteria that aided piglets confronting the stress of early weaning.

In our preliminary study, Yoshioka et al. (2015) performed the proximate analysis and microbial analysis of fermented taro skins from a local poi manufacturer. It was found that LAB dominated the microbial population (Figure 1). *Leuconostoc mesenteroides*, one of the strains isolated from the sample, had antimicrobial effect against tested bacterial strains. Muller et al. (2002) demonstrated that poi could support the growth of bacteriocin-producing bacteria. Additionally, utilization of cassava peels for animal feeding and supporting the growth of LAB has been investigated (Balogun and Bawa, 1997; Nwokoro, 2014; Olafadehan et al., 2012; Ukanwoko and Ibeawuchi, 2009). Although research involving fermented taro skins is very limited and even nutritive value of taro skins is not fully available, the development of fermented taro skins as a functional feed in animal production is promising. Moreover, the LAB isolated from post-processed taro peels, which survive pressure-cooking, have high potential to be applied in food production.
Figure 1. Bacterial population in fermented taro skins by genus

- Arthrobacter
- Exiguobacterium
- Wautersiella
- Lactococcus
- Leuconostoc
- Weissella
CHAPTER 3. MATERIALS AND METHODS

3.1 Sample collection

Cooked taro skins were collected from four poi manufacturers on Oahu and Maui. Three out of four manufacturers used wetland taros for poi making and only one of them used dryland taros. At least two samples were obtained from each manufacturer. The sample was stored at room temperature for 48 h to allow growth of microorganisms and fermentation of taro skins.

3.2 Isolation and identification of LAB

3.2.1 Isolation and purification of LAB

After fermentation, 25 grams of taro skins sample were homogenized for 1 min in 225 ml of 0.1% peptone water using the Stomacher® 400 Circulator (Seward, UK). The homogenate was serially diluted with peptone water. A volume of 0.1ml of serial dilutions was spread over the surface of plate count agar (PCA) and Man, Rogosa and Sharpes (MRS) agar. PCA was to enumerate the whole microbial community whereas MRS agar provided nutrients specifically suitable for growth of LAB. Through the comparison of microbial counts between PCA and MRS agar, the dominance of LAB in the sample could be confirmed. All media were prepared following the manufacturer’s instructions. A 48 h of incubation at 35°C was performed after plating. Colonies on the plates of appropriate dilution were enumerated subsequently. Representative colonies from each sample were selected for further purification. Selected colonies were streaked on MRS agar plates and incubated for another 48 h at 35°C. A single colony was picked from each plate as one isolate, while the plates with no growth were discarded. Each isolate was inoculated individually in 5 ml of MRS broth and incubated at 35°C for 24 h. The purified isolates were identified by combined use of random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and
16S rDNA sequencing.

3.2.2 RAPD-PCR

Total DNA was extracted from 50 µl of overnight culture of each isolate with DNeasy Blood & Tissue Kit (QIAGEN) per the protocol for Gram-positive bacteria provided by the manufacturer. DNA extracts were stored at -20ºC until being used as template for RAPD-PCR fingerprinting. RAPD-PCR is recognized as a rapid and reliable method to differentiate LAB strains isolated from fermented foods (Martin et al., 2009; Pulido et al., 2005; Rossetti and Giraffa, 2005). The M13 primer (5’-GAG GGT GGC GGT TCT-3’) was used for amplification of template DNA according to the protocol previously described by Pulido et al. (2005). The amplification was performed with the MJ Mini Thermal Cycler (Bio-Rad) in 25 µl of reaction mixture with 2 µl of sample DNA, 12.5 µl Master Mix (400 µM dNTPs, 3mM MgCl₂, and 1.25 U Taq DNA polymerase), 10 µl nuclease-free water, and 0.5 µl of 50 mM M13 primer. The amplification initiated with one cycle of 95ºC for 5 min, followed by 35 cycles of 94ºC for 1 min (denaturation), 40ºC for 20 s (annealing), ramp to 72ºC at 0.6ºC/s, and 72ºC for 2 min (extension). The final extension was carried out at 72ºC for 10 min. Five microliters of RAPD-PCR products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel in 0.5×TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) at 55V for 10 hours. The PCR Markers from Promega was used as a DNA ladder for reference. The gel was photographed with a digital gel imager (Fotodyne) after staining with SYBR Green. RAPD-PCR banding patterns of all LAB isolates were obtained and analyzed. Isolates with the same pattern were grouped and considered as the same strain. Representative strains from each group that displayed unique banding pattern were analyzed by electrophoresis again on a summary gel following the same procedure described above. All PCR reactions in this study included a negative control containing the same PCR mixture without template DNA, which was run in parallel to determine potential occurrence of false positive results.
3.2.3 Identification of LAB isolates

16S rDNA genes were amplified by PCR for species-specific identification of representative LAB strains. The reaction mixture (25 µl) consisted of 1 µl template DNA, 12.5 µl Master Mix, 11 µl nuclease-free water, 0.25 µl 50 mM 16S F primer (5'-GGA GAG TTT GAT CCT GGC TCA G-3') and 0.25 µl 50 mM 16S R primer (5'-TAT TAC CGC GGC TGC TGG CAC -3'). The 16S rDNA PCR program was 95ºC for 5 min, 30 cycles of 95ºC for 30 s, 62ºC for 30 s, and 72ºC for 45 s; and finally, 72ºC for 10 min. The resulting amplicons were loaded into a 1.5% agarose gel, electrophoresed at 100V for 35 minutes. The gel was stained with SYBR Green and visualized under UV light. Bands with expected size were considered positive samples.

Amplified DNA was cleaned of excess primers and dNTPs with ExoSAP-IT before sequencing. 5 µl PCR products were mixed with 2 µl of ExoSAP-IT reagent completely. The mixture was incubated at 37ºC for 15 min, followed by inactivation of ExoSAP-IT by heating to 80ºC for 15 min. Gene sequencing was then performed by the Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) at the University of Hawaii. LAB isolates were identified by Blasting (Basic Local Alignment Search Tool) their 16S rDNA sequences with those known sequences available in the National Center for Biotechnology Information (NCBI) GenBank.

Frozen stocks were prepared for all identified LAB strains by inoculating 0.5 ml overnight cell cultures into 0.5 ml sterile 30% glycerol and stored at -80ºC for further use. All cell cultures used in the following experiments were transferred from the frozen stock to MRS broth and incubated at 35ºC for 24 h.

3.3 In vitro assessment of probiotics potential of LAB isolates

3.3.1 Acid tolerance

To select potential probiotics from all representative LAB isolates, their survival in
low pH environment was evaluated, following the procedure of Prasad et al. (1998). Phosphate buffer saline (PBS) was prepared and adjusted to pH 3.0 by addition of 6 M HCl to simulate the acidic condition in the gut. Fresh LAB cultures were obtained by activating frozen cultures twice in order to ensure complete recovery of injured cells caused by freezing. Activated cultures were diluted with appropriate amount of MRS broth to achieve an optical density (OD$_{600}$) of 0.6-0.7. The optical density of diluted culture was measured by an Ultraspec 10 Cell Density Meter (GE Healthcare Life Sciences). LAB cultures were centrifuged at 2500 rpm for 10 min to harvest cell pellets. The supernatants were removed and pellets were washed once in sterile PBS (pH 7.4). Cells were resuspended in PBS (pH 7.4), and 0.5 ml of suspension was pipetted into 4.5 ml of sterile PBS at pH 3.0. The mixture was then incubated at 35°C for 3 h. 100 µl of mixture from each LAB strain were sampled at 0, 1, 2, and 3 h, and plated onto MRS agar. The number of viable cells was estimated after incubation at 35°C for 48 h. LAB strains that had little reduction in number after 3 h of incubation at pH 3.0 were chosen as candidates for further selection.

### 3.3.2 Bile tolerance

LAB strains exhibiting considerable resistance to low pH were screened for bovine bile (Oxgall powder, Fisher Scientific) tolerance subsequently. Tolerance of selected LAB strains against bile at different concentrations (0.25%, 0.5%, 0.75%, and 1%) was evaluated. Appropriate amounts of Oxgall powder was dissolved completely in 4.5 ml of fresh MRS broth to achieve various concentrations of bile. A $10^{-2}$ dilution of overnight cultures of LAB was made with peptone water. 100 µl of diluted cultures was inoculated into 10 ml of MRS broth containing varying content of bile and then incubated at 35°C. Viable counts after 5 h were obtained by spread plate method as described previously. Survival of tested strains was expressed in final concentration of bacteria as – (< 2.0 log CFU/ml), + (2.0-3.0 log CFU/ml), ++ (3.0-4.0 log CFU/ml), +++ (4.0-5.0 log CFU/ml), and ++++ (5.0-6.0 log CFU/ml).
3.3.3 Viability in bile after pre-exposure to acid

Promising LAB strains that were tolerant to both low pH and bile were examined for their viability in bile after pre-exposure to acid, following the procedure of Prasad et al. (1998) with slight modification. Overnight cultures were adjusted to OD$_{600}$ 0.8-1.0 with MRS broth. Cells were harvested from cultures and washed as mentioned above. Pellets from 0.5 ml of suspension were resuspended in the same volume of PBS at pH 3.0 before incubation at 35ºC for 2 h. Pre-exposed cells were collected and resuspended in 0.5 ml PBS (pH 7.4), then inoculated into 4.5 ml of MRS media with 0.5% or 1% of bile. Mixtures were incubated at 35ºC. Viable counts of LAB strains were determined by plating method at 0, 3, 6, and 9 h. Triplicates were done for the assay.

3.3.4 Bactericidal effect on S. Typhimurium and L. monocytogenes

Eight representative LAB isolates, including four strains of *Leuconostoc lactis*, two strains of *Leuconostoc mesenteroides*, one *Lactobacillus plantarum*, and one *Lactococcus lactis*, showed high tolerance to bile after pre-exposure to acid. *S. Typhimurium* and *L. monocytogenes* were used as indicator pathogens to investigate the antimicrobial activity of these eight LAB strains. Activated LAB cultures were inoculated into MRS broth and incubated at 30ºC for 24 h. Fresh cultures were centrifuged at 6,000 rpm for 5 min to obtain the supernatants. The supernatants were filtered through 0.2 µm syringe-driven membrane filters (Corning) aseptically, which were considered cell-free supernatants (CFS). Prior to use in experiments, 50 µl of frozen stock cultures of *S. Typhimurium* or *L. monocytogenes* were transferred into 5 ml of Tryptic Soy Broth (TSB), and incubated at 35ºC for 24 h. Cell pellets of 1 ml overnight cultures ($10^9$ CFU/ml) were obtained by centrifuging at 12,000 rpm for 1 min, and washed twice and resuspended in 1 ml MRS broth. Suspensions were serially diluted in MRS broth to get dilutions with cell concentration of $10^6$ CFU/ml. 0.2 ml of diluted indicator pathogen cultures were added separately into 4 ml CFS of tested LAB strains or MRS broth as control. The mixture was incubated at 39ºC. To determine the number of
surviving cells, sampling for enumeration was done at 0, 1, 2, 4, 6 h and 0, 2, 4, 8 h for *S. Typhimurium* and *L. monocytogenes*, respectively. All plates were incubated at 35°C for 24 h. The experiments were replicated three times.

### 3.4 Exploring the potential of bacteriocin production by LAB isolates

#### 3.4.1 Survival of indicator pathogens in neutralized CFS of LAB isolates

To verify the presence of antimicrobial substances other than organic acids in the metabolites of selected LAB isolates, the survival of indicator pathogens in neutralized CFS was investigated. The pH value of MRS broth is approximately 6.5 ± 0.2. CFS was prepared as described above, and its pH was adjusted to 6.5 using 3 M NaOH in order to eliminate the effect of organic acids. Neutralized CFS was filter sterilized again to avoid contamination during pH adjustment. Same procedures applied in the antimicrobial assay of original CFS were followed to investigate the susceptibility of *S. Typhimurium* and *L. monocytogenes* to neutralized CFS of LAB isolates.

#### 3.4.2 Detection of antimicrobial activity of protein-related substances

##### 3.4.2.1 Agar diffusion assay

In addition to organic acids, the presence of bacteriocin-like compounds was assumed to contribute to the antimicrobial activity of LAB isolates. To compare the antimicrobial effect of CFS of LAB isolates with and without proteinase treatment, the agar-well diffusion assay was employed. 10 µl of proteinase k (>600 AU/ml) were added into 40 µl CFS, and mixed thoroughly by vortexing. The mixture was incubated at 21°C for 30 min, and then placed in boiled water for 10 min to inactivate proteinase k, which is potentially inhibitory to the indicator pathogens. For the control, sterile distilled water was used to replace the proteinase k. 15 ml of PCA in a test tube as autoclaved and placed in a water bath to keep the temperature at 50°C, at which neither the agar would solidify nor the indicator bacteria would
be killed. 1 ml of the indicator bacteria at $10^5$ CFU/ml was added into PCA, mixed completely by swirling, and then poured into a sterile petri dish. The plates containing indicator bacteria were left at room temperature under laminar airflow for 1 h until the agar was completely solidified. Wells of 6 mm in diameter were punched into the plates and 50 µl of CFS with or without proteinase k treatment were loaded into each well. Plates were kept for 3 hours at room temperature to allow diffusion of CFS into the media, and then incubated at 35°C until clear inhibition zones appeared. The diameter of inhibition zones around the wells was measured, which indicated the antimicrobial effect on indicator pathogens. A smaller inhibition zone generated by proteinase k treated CFS than non-treated CFS implicates the presence of bacteriocin-like compounds with antimicrobial effect.

3.4.2.2 Spot-on-lawn assay

To further determine the antimicrobial activity of bacteriocin-like substances in the metabolites of selected LAB isolates, a spot-on-lawn assay was performed. The indicator, S. Typhimurium or L. monocytogenes, was grown overnight, and 100 µl of cultures were spread on the surface of dried MRS agar plates prepared in advance. The plates were placed under a laminar airflow system at room temperature for 30 min to allow the plates to dry. 20 µl of neutralized CFS was spotted onto the surface of the plates, and then kept at room temperature for diffusion of CFS. Positive antimicrobial activity of bacteriocins was evidenced after 24 to 48 h at 35°C as a clear inhibition zone around the spot on the lawn of indicator bacteria.

3.4.3 PCR-based detection of bacteriocinogenic LAB

The sensitivity of traditional agar well diffusion and spot-on-lawn assays was limited by many intrinsic and extrinsic factors. Thus, PCR-based detection methods were employed to detect bacteriocin-encoding genes in LAB isolates directly.
3.4.3.1 Detection of class IIa bacteriocin genes

Class IIa bacteriocin is one of the most significant groups of bacteriocins, which possess strong anti-*Listeria* activity and have been reported to contain a highly conserved YNGGVXCXXXCXV sequence motif at their N-terminus (Eijsink et al., 1989). Given that, a YNGGV-motif-based assay has been developed using 14 degenerate primers matching all class IIa bacteriocin-encoding genes currently available in the NCBI GenBank (Liu et al., 2014). In this study, class IIa bacteriocinogenic LAB were identified by YNGGV-motif-based PCR according to the procedure of Liu et al. (2014), using two pairs of primers, Clade 4-2 (forward: 5’- TGG AAA ATA TTA TGG AAA TGG AGTG -3’; reverse: 5’-CCT GGA ATT GCT CCA CCT AA -3’) and Clade 4-3 (forward: 5’-GAG ACA CAA CTT ATC TAT GGG GGTA- 3’; reverse: 5’-CCT GGA ATT GCT CCA CCT AA- 3’). Briefly, 25 µl PCR reactions were set up for amplification to target bacteriocin-encoding genes, containing 12.5 µl Master Mix, 10 µl nuclease-free water, 0.25 µl 50 mM Clade 4-2 F or Clade 4-3 F primer, 0.25 µl 50 mM Clade 4-2 R or Clade 4-3 R primer, and 2 µl of DNA extract from each LAB isolate. The temperature gradient PCR was performed under the following conditions: initial denaturation at 94ºC for 5 min, 30 cycles of denaturation at 94ºC for 45 s, annealing at a temperature gradient from 49-56ºC for 45 s, and extension at 72ºC for 1 min, followed by final extension at 72ºC for 5 min. The resulting PCR products were subjected to agarose gel electrophoresis (2.5%) at 100V for 35 min. The gels were photographed after staining with SYBR Green.

3.4.3.2 Detection of bacteriocin genes of *Lactococcus lactis* isolate

Based on the spot-on-lawn assay of neutralized CFS, inhibition zone was obtained against *L. monocytogenes* by only one *Lactococcus lactis* isolate (#137), demonstrating that this strain has high potential to produce anti-*Listeria* bacteriocins. Therefore, a bacteriocin-specific PCR array was employed using eight pairs of primers (Table 1) designed by Macwana et al. (2012) to target all known bacterocin structural genes of *Lactococcus lactis*. The PCR was performed
in a 25 µl reaction volume consisting of 12.5 µl Master Mix, 10 µl nuclease-free water, 0.25 µl of forward and reverse primers from an individual pair, and 2 µl of DNA extract from Lactoccus lactis #137. Nine PCR reactions were set up in total, including one 16S rDNA PCR as a positive control. The cycling procedures were as follows: initial denaturation at 94ºC for 5 min, 30 cycles each consisting of denaturation at 94ºC for 45 s, annealing at specific temperature range from 54-60ºC (see Table 1 for details) for 45 s, and extension at 72ºC for 1 min, followed by a final extension step at 72ºC for 5 min. Amplicons showing positive results in agarose gel electrophoresis analysis were sent out for sequencing in both directions. Amplicons sequences were analyzed to determine their similarity to bacterocin-encoding gene sequences available in the NCBI GenBank.
<table>
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<th>Size (bp)</th>
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<td><em>Lactococcus lactis</em> subsp. <em>cremoris</em></td>
<td>LacA-F</td>
<td>agtgctattcacaattctggcg</td>
<td>57.7</td>
<td>217</td>
</tr>
<tr>
<td></td>
<td>LacA-R</td>
<td>taacctcaccsccggtaaga</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> subsp. <em>cremoris</em></td>
<td>IcpJ-F</td>
<td>tggaccttatattttagttgcaaa</td>
<td>57.7</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>IcpJ-R</td>
<td>gacgacagtaaaatcagttcc</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>NisZ-F</td>
<td>atgagtaaaagatfatttaacttg</td>
<td>56.4</td>
<td>174</td>
</tr>
<tr>
<td></td>
<td>NisZ-R</td>
<td>ttattgtctacgtaataactaca</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> KF147</td>
<td>Bac147-F</td>
<td>accaatggtttgggtaactgta</td>
<td>54</td>
<td>314</td>
</tr>
<tr>
<td></td>
<td>Bac147-R</td>
<td>aaattaacctgttccacattc</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.5 Sensitivity of bacteriocin-like compounds to heating and enzymes

Neutralized CFS from *Lactococcus lactis* #137 was prepared by the same means as described above and divided into four equal portions, of which one was subjected to heating sensitivity test and the others were examined for sensitivity to enzymatic treatments. For heating sensitivity test, the neutralized CFS was heated in boiling water for 10 min. Other portions of the neutralized CFS were incubated in presence of 1 mg/ml catalase, proteinase K, or pronase E at 35°C for 2 h. Following the incubation, samples treated by various enzymes were heated at 100°C for 10 min to denature the enzymes and stop the reaction. The antimicrobial activities of the samples with heat or enzyme treatment were estimated by the survival of *L. monocytogenes* over a course of 48 h using the enumeration method described above. The neutralized CFS without any treatment was used as control. All experiments were performed in triplicate.
CHAPTER 4. RESULTS

4.1 Isolation and Identification of LAB

After presumptive LAB were isolated from fermented taro skins, they were purified on MRS agar. The banding pattern of each isolate was obtained by RAPD-PCR using M13 primer. Figure 2 exhibits the representative RAPD-PCR profiles. The profiles of all LAB isolates were compared to one another in order to differentiate isolates with unique binding patterns and group those with identical profiles as the same strain. Based on the results of RAPD-PCR analysis, one isolate from each group of strains was subjected to 16S rDNA sequencing. The isolates with negative results for 16S rDNA PCR were not considered bacteria and thereby excluded. All positive strains were identified by comparing their sequences of 16S rDNA to those available in GenBank using the BLAST Program (Table 2).

4.2 LAB community of fermented taro skins from different poi manufacturers

Table 3 lists the LAB species identified in all taro skin samples collected from four poi manufacturers. Eleven LAB species have been identified in total, consisting of two Lactobacillus species, two Lactococcus species, five Leuconostoc species, and two Weissella species. Leuconostoc was the most prevalent genus for all poi manufacturers, accounting for 80.5% of the LAB isolates. More specifically, L. mesenteroides was a predominant species in the samples from three poi manufacturers, whereas L. holzapfeli dominated the samples from manufacturer 4. Thus, Leuconostoc spp. were the main contributors to the antimicrobial activities of fermented taro skins. Moreover, La. lactis, L. lactis, L. pseudomesenteroides, and W. confusa, which occurred in samples from three or more poi manufacturers, might also play an important role in the inhibitory effect of fermented taro skins against pathogenic bacteria. No significant difference was observed in the LAB composition between wetland and dryland samples. Specific LAB species only found in one or two samples with low occurrence were probably originated from the unique environment where the poi was processed.
Figure 2. RAPD-PCR gel profiles for representative LAB isolates from fermented taro skins.
Table 2. Sequencing information for representative LAB isolates with unique binding patterns in RAPD-PCR

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Closest Relative</th>
<th>% Identity</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td><em>Leuconostoc lactis</em> strain</td>
<td>99%</td>
<td>NR_113255.1</td>
</tr>
<tr>
<td>#2, #6, #7, #12, #14-16</td>
<td><em>Lactococcus lactis</em> subsp. <em>lactis</em></td>
<td>97%</td>
<td>NR_103918.1</td>
</tr>
<tr>
<td>#3-5, #8, #10, #11, #17</td>
<td><em>Leuconostoc mesenteroides</em> strain</td>
<td>99%</td>
<td>NR_074957.1</td>
</tr>
<tr>
<td>#9, #13, #18</td>
<td><em>Leuconostoc pseudomesenteroides</em></td>
<td>99%</td>
<td>NR_040814.1</td>
</tr>
<tr>
<td>#19, #21, #25, #28, #32</td>
<td><em>Leuconostoc lactis</em></td>
<td>97%</td>
<td>NR_113255.1</td>
</tr>
<tr>
<td>#20</td>
<td><em>Weissella confusa</em></td>
<td>98%</td>
<td>NR_113255.1</td>
</tr>
<tr>
<td>#22, #24, #26</td>
<td><em>Leuconostoc pseudomesenteroides</em></td>
<td>99%</td>
<td>NR_042620.1</td>
</tr>
<tr>
<td>#23, #30</td>
<td><em>Leuconostoc holzapfelii</em></td>
<td>99%</td>
<td>NR_042620.1</td>
</tr>
<tr>
<td>#27, #31</td>
<td><em>Lactococcus lactis</em> subsp. <em>lactis</em></td>
<td>99%</td>
<td>NR_103918.1</td>
</tr>
<tr>
<td>#33</td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>96%</td>
<td>NR_074957.1</td>
</tr>
<tr>
<td>#34, #35, #37, #39-41, #46, #48-54, #56-65</td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>99%</td>
<td>NR_074957.1</td>
</tr>
<tr>
<td>#36, #55</td>
<td><em>Leuconostoc lactis</em></td>
<td>99%</td>
<td>NR_040823.1</td>
</tr>
<tr>
<td>#38, #43</td>
<td><em>Weissella soli</em> strain</td>
<td>99%</td>
<td>NR_025642.1</td>
</tr>
<tr>
<td>#42</td>
<td><em>Weissella confusa</em> strain</td>
<td>99%</td>
<td>NR_040816.1</td>
</tr>
<tr>
<td>#44</td>
<td><em>Lactococcus lactis</em></td>
<td>99%</td>
<td>NR_103918.1</td>
</tr>
<tr>
<td>#45</td>
<td><em>Lactococcus chungangensis</em></td>
<td>99%</td>
<td>NR_044357.1</td>
</tr>
<tr>
<td>#47</td>
<td><em>Leuconostoc pseudomesenteroides</em></td>
<td>99%</td>
<td>NR_040814.1</td>
</tr>
<tr>
<td>Isolate ID</td>
<td>Closest Relative</td>
<td>% Identity</td>
<td>GenBank Accession No.</td>
</tr>
<tr>
<td>------------</td>
<td>----------------------------------</td>
<td>------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>#71, #72, #74,</td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>93%</td>
<td>NR_074957.1</td>
</tr>
<tr>
<td>#95, #107, #125,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#127, #129, #132</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#73, #83, #84,</td>
<td><em>Leuconostoc pseudomesenteroides</em></td>
<td>92%</td>
<td>NR_042620.1</td>
</tr>
<tr>
<td>#85, #126, #128</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#76, #133, #135</td>
<td><em>Lactococcus lactis subsp. lactis</em></td>
<td>98%</td>
<td>NR_103918.1</td>
</tr>
<tr>
<td>#77</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#78</td>
<td><em>Leuconostoc pseudomesenteroides</em></td>
<td>93%</td>
<td>NR_042620.1</td>
</tr>
<tr>
<td>#86, #87, #96</td>
<td><em>Weissella confusa</em></td>
<td>98%</td>
<td>NR_040816.1</td>
</tr>
<tr>
<td>#89, #92, #98</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#90, #91, #94,</td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>98%</td>
<td>NR_074957.1</td>
</tr>
<tr>
<td>#121, #131,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#93</td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>98%</td>
<td>NR_074957.1</td>
</tr>
<tr>
<td>#97</td>
<td><em>Leuconostoc lactis</em></td>
<td>99%</td>
<td>NR_113255.1</td>
</tr>
<tr>
<td>#99, #100</td>
<td><em>Leuconostoc lactis</em></td>
<td>99%</td>
<td>NR_113255.1</td>
</tr>
<tr>
<td>#101</td>
<td><em>Lactobacillus plantarum</em></td>
<td>99%</td>
<td>NR_075041.1</td>
</tr>
<tr>
<td>#103, #110</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#102, #107, #112,</td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>99%</td>
<td>NR_074957.1</td>
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<tr>
<td>#115,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#104, #106</td>
<td><em>Leuconostoc pseudomesenteroides</em></td>
<td>92%</td>
<td>NR_042620.1</td>
</tr>
<tr>
<td>#105, #124</td>
<td><em>Leuconostoc citreum</em></td>
<td>99%</td>
<td>NR_074694.1</td>
</tr>
<tr>
<td>#108</td>
<td><em>Lactococcus lactis subsp. lactis</em></td>
<td>99%</td>
<td>NR_103918.1</td>
</tr>
<tr>
<td>Isolate ID</td>
<td>Closest Relative</td>
<td>% Identity</td>
<td>GenBank Accession No.</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------</td>
<td>------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>#111, #113, #114, #116-118, #120</td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>99%</td>
<td>NR_074957.1</td>
</tr>
<tr>
<td>#136, #167, #169, #172, #173</td>
<td><em>Leuconostoc holzapfelli</em></td>
<td>99%</td>
<td>NR_042620.1</td>
</tr>
<tr>
<td>#137</td>
<td><em>Lactococcus lactis subsp. lactis</em></td>
<td>98%</td>
<td>NR_103918.1</td>
</tr>
<tr>
<td>#138</td>
<td><em>Lactococcus lactis subsp. lactis</em></td>
<td>99%</td>
<td>NR_103918.1</td>
</tr>
<tr>
<td>#139, #140, #145, #147, #151, #155, #166</td>
<td><em>Leuconostoc pseudomesenteroides</em></td>
<td>99%</td>
<td>NR_109004.1</td>
</tr>
<tr>
<td>#141, #143, #144, #148, #149, #160, #162, #163, #165, #174, #175</td>
<td><em>Leuconostoc holzapfelli</em></td>
<td>99%</td>
<td>NR_042620.1</td>
</tr>
<tr>
<td>#142, #150, #188, #192</td>
<td><em>Lactococcus lactis subsp. lactis</em></td>
<td>99%</td>
<td>NR_103918.1</td>
</tr>
<tr>
<td>#146, #189</td>
<td><em>Lactococcus lactis subsp. lactis</em></td>
<td>99%</td>
<td>NR_103918.1</td>
</tr>
<tr>
<td>#152, #161, #164, #170, #171</td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>99%</td>
<td>NR_074957.1</td>
</tr>
<tr>
<td>#153</td>
<td><em>Leuconostoc lactis strain</em></td>
<td>99%</td>
<td>NR_113255.1</td>
</tr>
<tr>
<td>#154</td>
<td><em>Leuconostoc pseudomesenteroides</em></td>
<td>99%</td>
<td>NR_042620.1</td>
</tr>
<tr>
<td>#156-158</td>
<td><em>Lactobacillus nagelii</em></td>
<td>99%</td>
<td>NR_112754.1</td>
</tr>
<tr>
<td>#159</td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>99%</td>
<td>NR_074957.1</td>
</tr>
<tr>
<td>#168</td>
<td><em>Leuconostoc lactis</em></td>
<td>99%</td>
<td>NR_113255.1</td>
</tr>
</tbody>
</table>
Table 3. Species distribution of LAB isolated from 4 fermented taro skins

<table>
<thead>
<tr>
<th>Bacteria species</th>
<th>Poi (Wet-land)</th>
<th>Poi (Dry-land)</th>
<th>Manufacturer (Wet-land)</th>
<th>Manufacturer (Wet-land)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus nagelii</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em></td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td><em>Lactococcus chungangensis</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td><em>Leuconostoc citreum</em></td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><em>Leuconostoc holzapfelli</em></td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td><em>Leuconostoc lactis</em></td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>8</td>
<td>19</td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides</em></td>
<td>24</td>
<td>17</td>
<td>15</td>
<td>7</td>
<td>63</td>
</tr>
<tr>
<td><em>Leuconostoc pseudomesenteroides</em></td>
<td>0</td>
<td>5</td>
<td>8</td>
<td>11</td>
<td>24</td>
</tr>
<tr>
<td><em>Weisella confusa</em></td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td><em>Weisella soli</em></td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>38</td>
<td>36</td>
<td>54</td>
<td>159</td>
</tr>
</tbody>
</table>
4.3 In vitro assessment of probiotics potential of LAB isolates

4.3.1 Acid tolerance

Acid tolerance is required for the bacteria to survive passage through the stomach. Although the pH of stomach fluid can be as low as 1, pH 3 is more commonly used for in vitro study of LAB regarding acid resistance (Prasad et al., 1998). The time taken for digestion in the stomach is about 3 h (Hawaz et al., 2014). According to that, a total of 27 LAB isolates were examined for their survival at pH 3 in three hours by enumeration method (Figure 3). They exhibited different response to the acid environment. Most of strains were able to survive within 1 h, but only a few of them were still viable at 3 h. Overall, L. lactis showed the highest resistance to acid among all tested species, for which very little reduction in viable counts was observed after 3 h of treatment. There were also some strains from other species that were tolerant to low pH environment, such as strains #9, #29, #101 and #137, which were L. pseudomesenteroides, L. mesenteroides, L. plantarum, and La. lactis respectively. Eleven LAB isolates with relatively high tolerance to acid were chosen for further test on bile tolerance.

4.3.2 Bile tolerance

Bile in the small intestine possibly inactivates LAB before they arrive at the large intestine, where they colonize and confer health benefits to the host. The concentration of bile in the small intestine is suggested as 0.3% and the staying time is about 4 h (Parad et al., 1998). For bile tolerance assay, 11 selected acid-tolerant LAB isolates were incubated for 5 h in the presence of 0.25%, 0.5%, 0.75%, and 1% bile salts. Table 4 summarizes the survival of tested strains in bile by manufacturer. After 5 h of incubation, all tested strains survived 0.25% bile. They were also tolerant to 0.5% bile except isolate L. lactis #1. However, increased bile concentrations led to death of most strains. Eventually, only four isolates demonstrated tolerance to 0.75% and 1% bile, which were L. plantarum #101, L. lactis #97, La. lactis # 137 and L. mesenteroides #67. The isolates L. plantarum #101 and L.
*mesenteroides* #67 showed the strongest tolerance to bile. Actually, *L. plantarum* #101 even grew in the presence of bile.
Figure 3. Survival of representative LAB isolates in PBS at pH 3
Figure 3. (Continued) Survival of representative LAB isolates in PBS at pH 3
Table 4. Tolerance of LAB isolates to different concentrations of bile

<table>
<thead>
<tr>
<th>Source</th>
<th>LAB isolate b</th>
<th>Bile concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.25%</td>
</tr>
<tr>
<td>Manufacturer 1</td>
<td><em>L. lactic</em> #1</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td><em>L. plantarum</em> #101</td>
<td>++++</td>
</tr>
<tr>
<td>Manufacturer 2</td>
<td><em>L. pseudomesenteroides</em> #9</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>L. lactis</em> #12</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>L. lactis</em> #97</td>
<td>++</td>
</tr>
<tr>
<td>Manufacturer 3</td>
<td><em>L. lactis</em> #19</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>W. confusa</em> #20</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>L. lactis</em> #21</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td><em>L. mesenteroides</em> #29</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td><em>Lactis</em> #137</td>
<td>++</td>
</tr>
<tr>
<td>Manufacturer 4</td>
<td><em>L. mesenteroides</em> #67</td>
<td>+++</td>
</tr>
</tbody>
</table>

*++++*: 5.0-6.0 log CFU/ml; +++*: 4.0-5.0 log CFU/ml; ++*: 3.0-4.0 log CFU/ml; +*: 2.0-3.0 log CFU/ml; –: < 2.0 log CFU/ml

The original concentration of bacteria was 4.0-5.0 log CFU/ml.
4.3.3 Survival of LAB isolates in bile after pre-exposure to acid

After screening based on acid and bile tolerance, five promising LAB isolates, including two strains of *L. lactis*, one strain of each *L. mesenteroides*, *L. plantarum*, and *La. lactis*, were subjected to evaluation on the fate in MRS containing 0.5% and 1% bile after 2 h of pre-incubation in PBS at pH 3. All strains were capable of surviving both 0.5% and 1% bile treatments after 9 hours, except *L. lactis* #21. Basically, the survival patterns obtained in 0.5% and 1% bile were very similar for most strains. The strain (#101) that could grow in MRS with 0.5% bile also grew in 1% bile, and those strains that were inhibited in 1% bile also decreased in numbers in 0.5% bile. *L. plantarum* #101 was the most resistant strain in this assay, which grew gradually during the treatment and achieved a 2 logs increase in number within 9 h. Apparently, the pre-exposure treatment did not affect its high resistance to bile. Therefore, *L. plantarum* #101 is a very promising strain which might survive the digestive tract and colonize in the gut. Eventually, four strains from four different LAB species (#67, #97, #101, and #137) were selected for further testing as potential probiotics. However, isolates that are tolerant to acid and bile in vitro are not necessarily able to survive in vivo since the environment in the gastrointestinal tract is much more complicated. Therefore, more experiments are needed to confirm the ability of these LAB strains to colonize in the gut and offer health benefits.
Figure 4. Effect of pre-exposure to pH 3 on the fate of LAB isolates in MRS broth containing 0.5% bile (A) and 1% bile (B).
4.4 Antimicrobial activity of LAB isolates against *Salmonella* and *Listeria monocytogenes*

In addition to the four most promising LAB isolates, the bactericidal activities of four other strains of *Leuconostoc* against *Salmonella Typhimurium* and *Listeria monocytogenes* have been studied. The survival of two indicator bacteria in LAB supernatants is illustrated in Figures 5 and 6. The ability of tested LAB to inhibit two pathogens varied by the strain and the type of indicator organism. Isolates #97 (*L. mesenteroides*) and #101 (*L. plantarum*) exhibited the strongest bactericidal effect on *S. Typhimurium*, for which inactivation was achieved in only 1 h (5 log reduction). #67 (*L. mesenteroides*) and #137 (*La. lactis*) were slightly less effective strains, which killed the pathogen in 2 h. As for *L. monocytogenes*, isolate #101 (*L. plantarum*) was the most effective LAB, inactivating the indicator in 2 h. Moreover, no viable cells were observed in treatment of #97 at 4 h. The two strains that exhibited the weakest bactericidal effect were #21 (*L. lactis*) and #29 (*L. mesenteroides*), only leading to a 1-log reduction in *L. monocytogenes* counts after 4 h, though death of the indicator occurred by 8 h. It was observed that *S. Typhimurium* died in 4 h with treatments of all LAB strains, which indicates *S. Typhimurium* is more sensitive to tested LAB strains than *L. monocytogenes*. This finding is consistent with a previous study reporting that *Salmonella* was particularly sensitive to LAB supernatants (Anyogu et al., 2014).
Figure 5. Survival of *S. Typhimurium* in LAB culture supernatants

Figure 6. Survival of *L. monocytogenes* in LAB culture supernatants
4.5 Exploring the potential of bacteriocin production by LAB isolates

4.5.1 Effect of proteinase K treatment on the antimicrobial effect of LAB

Bacteriocins are protein-related substances, whose proteinaceous nature has been verified by previous research (Sip et al., 2015; Sriorntual et al., 2007). It was reported that the anti-
*Listeria* substances lost the inhibitory activity after treatment with proteinase K (Chopra et al., 2014; Sip et al., 2015; Sriorntual et al., 2007). To evidence the presence of bacteriocin-like antimicrobials in the metabolites of LAB isolates, the inhibitory effect of CFS treated with proteinase K was evaluated by agar well diffusion assay (Figures 7 and 8). Inhibition zones against *S. Typhimurium* obtained by CFS of LAB isolates with and without proteinase K were similar in size, indicating that the antimicrobials inhibitory against *Salmonella* were not sensitive to proteinase K.

However, smaller inhibition zones against *L. monocytogenes* were observed in the cases of #1, #19, #21, #29, and #137 with treatment by proteinase K, comparing to the controls without enzyme treatment. The decreased size of inhibition zones was probably attributed to the degradation of antimicrobial substances by proteinase K. This suggests the potential presence of bacteriocin-like antimicrobials in the CFS.
Figure 7. Size of inhibition zones against *S. Typhimurium* obtained by cell free supernatants of LAB isolates with and without proteinase K treatment.

Figure 8. Size of inhibition zones against *L. monocytogenes* obtained by cell free supernatants of LAB isolates with and without proteinase K treatment.
4.5.2 Effect of neutralization on the antimicrobial effect of LAB isolates

Organic acids are known as the major bactericidal substances produced by LAB, which mainly account for their inhibitory effect on pathogenic bacteria. To further study the nature of other antimicrobial components produced by LAB isolates, the acids were excluded from the supernatants of LAB isolates. The neutralized cell free supernatants (CFS) were examined for the antimicrobial activities against *S. Typhimurium* and *L. monocytogenes*. Agar diffusion assay did not yield any inhibition zone. In spot-on-lawn assay, no inhibition zone occurred on the lawn of either *S. Typhimurium* or *L. monocytogenes* with all treatments except *La. lactis* #137. Figure 9 shows an inhibition zone against *L. monocytogenes* achieved by neutralized CFS of #137.

Furthermore, the enumeration assay was performed for more convincing evidence. The viable cells of two indicator bacteria in the neutralized CFS were enumerated over time by spread plating method. *S. Typhimurium* incubated in the neutralized supernatants of LAB isolates grew as fast as in the control, in which the viable counts increased from 5 log CFU/ml to around 9 log CFU/ml within 24 h (Figure 10). The numbers of viable cells obtained in neutralized CFS of some strains of LAB were even slightly higher than those in the control. According to that, neutralization of acids eliminated the antimicrobial effect of LAB isolates on *S. Typhimurium*.

However, a different situation has been observed in the assay against *L. monocytogenes*. Although considerable growth of *L. monocytogenes* occurred in neutralized CFS of most tested strains, isolate #137 caused a more than 4 log reduction by 24 h and the pathogen died within 48 h (Figure 11). This agrees with the result of the spot-on-lawn assay. Bacteriocins have been reported to have relatively narrow spectra of bactericidal activity (De Vuyst and Leroy, 2007). Bacteriocins produced by LAB frequently inhibit closely related Gram-positive bacteria. Thus, it could be illustrated that isolate #137 retained the bactericidal effect on *L. monocytogenes* after exclusion of acids due to the production of other antimicrobials, which most probably were bacteriocin-like compounds.
Figure 9. Inhibition zone on the lawn of *L. monocytogenes* obtained by neutralized cell free supernatant of *La. lactis* #137
Figure 10. Viable count of *S. Typhimurium* in neutralized CFS of LAB isolates

Figure 11. Viable Count of *L. monocytogenes* in neutralized CFS of LAB isolates
4.6 PCR-based detection of bacteriocin-encoding genes

4.6.1 Detection of class IIa bacteriocin genes

The low sensitivity of traditional detection methods lowers the possibility to identify bacteriocinogenic LAB isolated from fermented taro skins. The results of proteinase K and neutralization treatment tests implicated the production of bacteriocins by some strains, particularly #137. Class IIa bacteriocins are small heat-stable peptides with high anti-
Listeria activity that have attracted increasing interest. Because of their wide distribution and anti-
Listeria characteristic, we assumed that class IIa bacteriocins are the bacteriocin-like substances present in the CFS of LAB isolated from fermented taro skins. Thus, a PCR-based approach developed by previous research was adopted to directly amplify the bacteriocin-encoding genes carried by class IIa bacteriocinogenic strains (Więckowicz et al., 2011). Gradient PCR aiming at class IIa bacteriocin-encoding genes was performed on DNA extracts from each identified LAB strains. However, no positive result was obtained with the gel electrophoresis analysis of PCR products, which denied the presence of class IIa bacteriocin genes in any LAB isolates.

4.6.2 Detection of bacteriocin genes carried by LAB isolate #137

Although no class IIa bacteriocin gene was detected, isolated LAB might have the potential to produce bacteriocins in other classes rather than IIa. Primers designed based on conserved sequence motifs shared by class IIa bacteriocin coding genes are not applicable for PCR-based detection of bacteriocins in other classes. Based on the results of previous experiments, isolate #137, a strain of La. lactis, was the one most likely to produce anti-
Listeria bacteriocins among all selected strains. Therefore, eight pairs of primers (Table 1), representing all known structural genes of bacteriocins of La. lactis in the NCBI GenBank (Macwana et al., 2011), were employed in PCR-based detection of bacteriocin-encoding genes for #137. Amplicons were successfully generated by two sets of primers designed on two different bacteriocin genes, NisZ (Lactococcus lactis) and Bac147 (Lactococcus lactis KF147). Primers NisZ generated a final PCR product of about
180 bp and Primers Bac147 gave a product of about 320 bp, of which both were at the expected molecular sizes (Figure 12). By comparing the sequencing results from two different directions, two full DNA sequences of 176 bp and 291 bp, amplified by primers NisZ and Bac147 respectively, were obtained.

The DNA sequences were analyzed by a BLAST search in the GenBank, and the identification results are shown in Table 5. When the DNA sequence generated by primers Bac147 was compared with that of *La. lactis* KF147, there were obvious differences between the intra-primer region of #137 and that pertaining to KF147. However, high homology to that of *La. lactis* strain Al06 genome was observed (Figure 13). In spite of the high identity, there is one nucleotide difference between the amplified DNA sequence and *La. lactis* strain Al06 gene. Primers NisZ generated sequence was found to be identical to sequences of several nisin genes. To our knowledge, this is the first report on *Lactococcus lactis* strain containing these two bacteriocin structural genes simultaneously, which makes the isolate #137 a potential novel bacteriocinogenic LAB.
Figure 12. Agarose gel electrophoresis of PCR amplicons generated from total DNA of isolate #137 with primers NisZ and Bac147 for *La. lactis* related bacterocin genes. Lane M: molecular weight marker. Primers 16S rDNA was used as control to assure the quality of the template DNA.

Table 5. Identification of isolate #137 with bacteriocin structural genes amplified by primers Bac147 and NisZ

<table>
<thead>
<tr>
<th>Primers</th>
<th>Closest Relative</th>
<th>Identity</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bac147</td>
<td><em>Lactococcus lactis</em> strain Al06</td>
<td>99%</td>
<td>CP009472.1</td>
</tr>
<tr>
<td>NisZ</td>
<td><em>Lactococcus lactis</em> subsp. <em>lactis</em> CV56</td>
<td>100%</td>
<td>CP002365.1</td>
</tr>
</tbody>
</table>
Figure 13. Sequence analysis of amplicons from *La. lactis* #137. A, alignment of identified bacteriocin gene sequence with the original bacteriocin gene sequence (KF147) on which the primers were designed. B, alignment of identified bacteriocin gene sequence with the sequence of the most relative gene of *La. lactis* strain Al06.
4.7 Sensitivity of bacteriocins produced by isolate #137 to heating and enzymes

In order to further characterize the anti-
Listeria substances produced by isolate #137, the influence of heating and several enzymes on the inhibitory activity was investigated (Figure 14). Like most bacteriocins, the anti-
Listeria substances in neutralized CFS of isolate #137 were heat resistant, whose activity was not affected by heating at 100ºC for 10 min. The assay has also shown that 2 h of treatment with proteinase K or pronase E eliminated the inhibitory effect of the antimicrobials, whereas the catalase had no effect on the inhibitory effect. The catalase sensitivity test excluded the presence of hydrogen peroxide in the CFS. Overall, the sensitivity to proteinase K and pronase E verified the proteinaceous nature of the anti-
Listeria substances produced by #137.

Figure 14. Survival of \textit{L. monocytogenes} in neutralized CFS after treatment with heating, catalase, proteinase K and pronase E
CHAPTER 5. DISCUSSION

The purposes of this study were to identify the predominant LAB species in fermented taro skins and to determine the probiotic potential and antimicrobial activity of the isolates. A total of 159 isolates belonging to 11 different LAB species were isolated from four sources and identified based on RAPD-PCR and the 16S rDNA sequencing, of which majority were *Leuconostoc* spp. Our results confirm the predominance of *Leuconostoc* spp. in fermented taro skins, which has been demonstrated by Yoshioka et al. (2015), regardless of the source of the samples. The presence of *Leuconostoc* spp. with antimicrobial activity has been reported in various fermented foods, including vegetables, dairy products, sausage, and wine (Albesharat et al., 2011; Drosinos et al., 2005; Héchard et al., 1992; Masuda et al., 2011; Montersino et al., 2008). *L. mesenteroides* strains accounted for approximately 40% of all isolates, and their strong antimicrobial effects exhibited in the following assays. Thus, *L. mesenteroides* was a crucial species responsible for the antimicrobial activity of fermented taro skins. *Lactococcus* and *Lactobacillus* have been reported as dominant bacterial species in sour poi in two studies (He, 2003; Huang et al., 1994). However, only a small number of isolates in these two genera were obtained from our samples. Furthermore, several LAB species, that have not been reported to be present in poi, were isolated from the samples, including *L. nagelii, L. holzapfeli*, and *L. pseudomesenteroides*. These species have been associated with fermentation of wine, coffee, and cocoa, respectively (De Bruyne et al., 2007; Edwards et al., 2000; Schwan and Fleet, 2014).

Probiotic potential of the identified strains was evaluated in this study. All LAB isolates were obtained from fermented taro skins removed from cooked taro, which has established the safety of them for consumption. On the other hand, for the selection of strains with health-promoting effects, it is important that the bacteria can survive and establish themselves under the stressful conditions encountered in the gastrointestinal environment. Exposure to PBS at pH 3 for 3 h was performed to evaluate the acid tolerance of representative strains. This screening method for acid tolerance has been validated by other researchers (Conway et al., 1987; Prasad et al., 1998). Although the pH of stomach
fluid can be as low as 1, probiotics are usually consumed with food as a carrier matrix, which can buffer the environment protecting the probiotics from high acidity in the stomach (Prasad et al., 1998). *L. lactis* was the species that had the highest tolerance to low pH, whereas specific isolates in other species also survived. About half of the isolates were eliminated for further screening due to their sensitivity to acid.

Bile concentrations between 0.1% and 4% (w/v) in growth media have been commonly used in bile tolerance assay, while the mean concentration of bile in the intestine is estimated at 0.3% (Gilliland et al., 1984). In this study, low-pH tolerant strains were examined for their tolerance to 0.25%, 0.5%, 0.75% and 1% of bile in MRS media. Almost all tested strains survived 0.25% and 0.5% bile, but most of them were killed in the higher concentrations. Only stains #67, #97, #101 and #137 were tolerant to high bile contents. The following bile tolerance assay after pre-exposure to pH 3 confirmed strong potential of these four strains to survive in the gastrointestinal tract. It is worth to mention that a 2 log growth was observed on isolate #101 *L. plantarum*, when it was incubated in MRS containing 1% bile after the low-pH treatment. This strain might have great advantages *in vivo*. Other *in vitro* methods have been employed to screen probiotic strains for their survival in the gut, such as the use of gastric juice and a dynamic model of stomach and small intestine (Conway et al., 1987; Marteau et al., 1997; Xanthopoulos et al., 2000). These methods provide conditions more similar to the gastrointestinal environment, but they are more complicated and costly.

Although various LAB species in poi have been isolated and identified in previous research (Allen and Allen, 1933; Muller et al., 2002; He et al., 2003; Huang et al., 1994), their ability to inhibit pathogenic bacteria remained to determine. Meanwhile, very limited information about fermented taro skins is available. Selected LAB strains isolated from fermented taro skins exhibited very high antimicrobial activities against *S. Typhimurium* and *L. monocytogenes*, two typical foodborne pathogens causing serious problems in the food industry and animal production. The decrease of inhibition zones against *L. monocytogenes* after treatment of CFS with proteinase K indicated the production of protein-related anti-*Listeria* compounds by isolates #1, #19, #21, #29, and #137, though acids were the main antimicrobial factors. However, the results of agar-well diffusion and
spot-on-lawn assays demonstrated that neutralization invalidated the inhibitory effect of the supernatants of those LAB except isolate #137 *La. lactis*. These results were in accordance with the following broth enumeration assay. The pH of supernatants of all tested strains ranged from 3.70 to 4.35, which was adjusted to 6.5 during neutralization. The antimicrobial activity of certain bacteriocins was reported to be pH-dependent and highly active only in acidic conditions (Houlihan et al., 2006; Mortvedt-Abildgaard et al., 1995; Rayman et al., 1993). According to Mortvedt-Abildgaard et al. (1995), lactocin S had a pH threshold of 6, above which no bactericidal effect was observed probably due to the loss of cationic nature of lactocin S. Houlihan et al. (2006) found bovicin HC5 was highly stable in acidic environments and bound to target bacteria only at pH values less than 6.0. The mechanisms behind the influence of pH change on the activity of bacteriocins have not been well defined. Although the proteinase K treatment assay suggested the production of bacteriocins by isolates #1, #19, #21 and #29, their antimicrobial activity might have lost after neutralization due to sensitivity to environmental pH. On the other side, both spot-on-lawn and broth enumeration assays confirmed that isolate #137 could produce bacteriocins against *L. monocytogenes* and the activity retained at pH 6.5.

The use of agar well diffusion and spot-on-lawn assays to detect or characterize bacteriocins have been documented in many reports (Anyogu et al., 2014; Lejeune et al., 1998; Krier et al., 1998; Touré et al., 2003; Verluyten et al., 2004). The sensitivity of these traditional approaches was relatively low according to our experience, probably attributed to limited ability of bacteriocins to diffuse through the agar (Wolf and Gibbons, 1996). Consequently, molecular techniques with much higher sensitivity are necessary to confirm the results obtained by traditional culture-based methods.

No class IIa bacteriocin gene was amplified by YGNGV-motif-based gradient PCR, implicating that the bacteriocin-like compounds produced by isolates #1, #19, #21, #29, and #137 belong to other classes rather than IIa. Because of the difficulty of detecting bacteriocins in various classes simultaneously, it was decided to focus on the LAB strain that showed the highest possibility of producing anti-*Listeria* bacteriocins, isolate #137. This isolate was identified as *La. lactis* by 16S rDNA sequencing. A bacteriocin PCR array established by Macwana et al. (2012) was performed to amplify specific bacteriocin
structural genes carried by the isolate. Two amplicons were obtained with primer pairs NisZ and Bac147, which target two different bacteriocin structural genes. Sequence analysis illustrated that the two amplicons had little homology to each other. The sequence generated by primers NisZ was short and identical to a few of nisin-encoding genes of La. lactis. Another sequence amplified by primers Bac147 has high homology (99%) to Lactococcus lactis strain Al06, instead of Lactococcus lactis KF147 (89% in homology), which the primers were derived from.

Lactococcus lactis KF147 is a LAB strain carrying a nisin structural gene yet shows no bacteriocin activity according the Kelly et al. (1998). In another word, bacteria possessing bacteriocin structural genes are not necessarily able to express the genes or produce bacteriocins. Isolate #137 was more similar to Lactococcus lactis strain Al06 than Lactococcus lactis KF147. Lactococcus lactis strain Al06 was isolated from the açaí fruit in Brazil. It has known probiotic properties which might contribute to the known benefits of açaí consumption (JGI 2014). Further test indicated that bacteriocins produced by #137 are tolerant to heating and catalase treatment but sensitive to proteinase K and pronase E treatment, similar to most of bacteriocins in class I. No previous report involving Lactococcus lactis strain Al06 has suggested its bacteriocin-producing property. Therefore, more work is needed to determine whether the bacteriocin production by isolate #137 is the consequence of expression of both genes or the gene amplified by NisZ primers only. The presence of multiple bacteriocin structural genes in the same strain has been reported in recent years (Henning et al., 2015; Macwana et al., 2012; Rehaiem et al., 2014). Nevertheless, this is the first report on the occurrence of these two bacteriocin structural genes in the same Lactococcus lactis strain.

Lactococcus lactis has been extensively used in fermentation of dairy products. Therefore, most well studied bacteriocin-producing La. lactis were obtained from dairy products (Siezen et al., 2010). Fermented plant materials are the second important source of La. lactis. Nisin- and other bacteriocin-producing La. lactis strains have been isolated from fermented or fresh vegetables previously (Choi et al., 2000; Han et al., 2013; Zendo et al., 2006). Isolate #137 is the first bacteriocin-producing La. lactis found in fermented taro skins. Furthermore, regardless of production of bacteriocins, several LAB strains identified
in this study hold high potential to be developed as probiotics with valuable application in food and animal production. *In vivo* study is going to be performed using animal models in other to determine if these strains are able to exert their health promoting effects in real gastrointestinal environment. In addition, the application of bacteriocins as a safe and effective weapon to control pathogenic bacteria occurring in food products and food animals has been demonstrated by other researchers (Davies et al., 1997; Foegeding et al., 1992; Han et al., 2013; Svetoch et al., 2010; Vignolo et al., 1996; Wright et al., 2010). Large-scale production of the newly discovered bacteriocins and their antimicrobial activities in different food matrices are also going to be explored in future.
CHAPTER 6. CONCLUSIONS AND FUTURE WORK

This study identified *Leuconostoc* spp. as the major component of microflora in fermented taro skins, which showed strong inhibitory effect on typical foodborne pathogens such as *Salmonella* Typhimurium and *Listeria monocytogenes*. Furthermore, several isolates exhibited high tolerance to low pH and bile, thereby enabling them to survive in the gastrointestinal environment. The adhesion of bacteria to intestinal cells is another important criterion for selection of probiotics. This property of the promising LAB isolates will be determined with human enterocyte-like Caco-2 cells later.

The antimicrobial activities of isolated LAB were mainly attributed to the production of organic acids, whereas bacteriocin-like substances with anti-*Listeria* activity were detected. The neutralization treatment potentially inactivated the bacteriocins produced by certain LAB isolates. More work is needed to confirm the presence of bacteriocins whose antimicrobial activity is pH-dependent. PCR-based methods are more reliable, and they directly detect bacteriocin-encoding genes in LAB. No class IIa bacteriocin gene has been detected in this study. Two different bacteriocin structural genes were amplified by two sets of primers within isolate #137 *La. lactis*. One was identified as nisin-encoding gene, while the other remains unknown. This is the first time to reveal a *La. lactis* strain containing these two bacteriocin genes. The anti-*Listeria* bacteriocins produced by the *La. lactis* strain were heat-stable but sensitive to proteinase K and pronase E. Application of the bacteriocins to inhibit pathogenic bacteria in food products and animal production would offer a very promising biocontrol tool.

Detection of bacteriocins potentially produced by other promising LAB isolates is ongoing. The collection and analysis of fermented taro skins from other poi manufacturers in Hawaii will continue. The LAB isolates that showed high acid and bile tolerance will receive more selection experiments. The health-promoting effects of promising LAB strains will be evaluated *in vivo* using animal models.
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