Norwegian University
of Life Sciences

Master's Thesis 2018 60 ECTS
Faculty of Chemistry, Biotechnology and Food Science

Screening and characterization of bacteriocins produced by lactic acid bacteria against *Lactococcus garvieae*

Lalaina Holivololona
Biotechnology, Microbiology

Acknowledgment

The work presented in this master's thesis was carried out at the Laboratory of Microbial Gene Technology (LMG), at the Norwegian University of Life Sciences (NMBU) from August 2017 to Mai 2018.

First of all, I would like to thank my main supervisor Dzung Bao Diep for letting me work on my master's project at LMG. Your guidance, advises, motivating words and cheerful jokes were much appreciated.

I would also like to express my gratitude to my co-supervisor Morten Kjos. Thank you for your help in the interpretation of the results, for your comments and suggestions, and for being so helpful in the whole process.

I am also thankful to several members of the laboratory. Thank you, Amar Telke for helping me in every step of the project, for your valuable advises and for assisting me in the utilization of some instruments. I must also express my gratitude to Kirill Ovchinnikov for helping me with the purification of the bacteriocin, for sharing valuable information, for small talks. Thank you Juan José Jiménez Martínez for your good mood, your support and advises.

Finally, I would like to thank my boyfriend, family and friends for their support, love, trust and encouragement.

Ås, May 2018

Lalaina Holivololona

Abstract

Infectious diseases caused by bacteria have ravaged humankind multiple times in history until the discovery of antibiotics. Now, several decades later, bacteria resistant to nearly all antibiotics have been reported due to the overuse and misuse of the latter. To remediate the alarming situation and avoid a disastrous future, novel approaches to antibiotics must be investigated. Bacteriocins are good candidates for the treatment of bacterial infections for several reasons. First, they have a narrow spectrum of activity compared to antibiotics, which limit the selective pressure for resistance to the pathogens instead of all the commensal bacteria. Bacteriocins are also non-toxic to humans since many are already used as food preservatives. Finally, they could be effective to fight antibiotic resistant bacteria due to their different killing mechanism.

The purpose of this study was to isolate and characterize lactic acid bacteriocins that could inhibit the fish and emerging human pathogen *Lactococcus garvieae*. To accomplish that goal, 50 samples of fermented fruits and vegetables were screened against *L. garvieae* by using a "sandwich overlay" method. The potential bacteriocin-producers were then identified by 16S rRNA gene sequencing and REP PCR profiling. To characterize the antimicrobials produced by the isolates, proteinase K and heat stability tests were conducted, and the inhibition spectrum was determined. Based on the results of these experiments, seven different strains were selected, and their genomes were sequenced on an Illumina Miseq system. The sequenced genomes were then uploaded on BAGEL4 to look for bacteriocin genes. The results showed that each genome contained putative bacteriocin genes and in some cases, more than one bacteriocin was identified. In addition, the observation that the identified bacteriocins belonged to different classes illustrates well the diversity of lactic acid bacteriocins.

The last experiment was the purification of the most relevant bacteriocin in respect to the purpose of the study. In that context, the bacteriocin from *Enterococcus thailandicus* was purified by ammonium sulfate precipitation followed by one reverse-phase chromatography step. The molecular mass was determined to be 6312 Da by MALDI-TOF MS, which confirms that the purified bacteriocin was the circular thaiocin 1.

The results of the study suggest the use of thaiocin 1 from *E. thailandicus* to control the growth of *L. garvieae* in fish farming.

Sammendrag

Infeksjonssykdommer forårsaket av bakterier har herjet menneskeheten flere ganger i historien, til oppdagelsen av antibiotika. Nå, flere tiår senere ser det ut som at bakteriene har kontrollen igjen grunnet observasjoner av bakterier som er resistente mot alle typer antibiotika. Overforbruk og misbruk av sistnevnte førte til den resistens-krisen i dag. For å rette opp den alarmerende situasjonen og unngå en katastrofal fremtid, må nye tilnærminger til antibiotika undersøkes.

Bakteriociner er gode kandidater for behandling av bakterielle infeksjoner av flere grunner. For det første har de et smalt spekter av antimikrobiell aktivitet sammenlignet med antibiotika, noe som begrenser det selektive trykket for resistens mot patogener istedenfor alle kommensale bakterier. Bakteriociner er også ikke-giftige for mennesker, ettersom mange er allerede brukt som mat konserveringsmidler. Til slutt kan de være effektive for å bekjempe antibiotikaresistente bakterier på grunn av deres ulike drepemekanisme.

Hovedmålet med denne oppgaven var å isolere og karakterisere bakteriociner produsert av melkesyrebakterier, som kunne hemme veksten av fiskepatogenen og fremvoksende humanpatogenen *Lactococcus garviea*e. For å oppnå målet ble 50 prøver av fermentert frukt og grønnsaker screenet mot *L. garvieae* ved å bruke en "sandwich overlay" metode. De potensielle bakteriocin-produsentene ble deretter identifisert med 16S rRNA-gen-sekvensering og REP-PCR-profilering. For å karakterisere de antimikrobielle forbindelsene produsert av isolatene ble proteinase K og varmestabilitet tester utført, og inhiberingsspekteret ble undersøkt. Basert på resultatene av disse undersøkelsene ble det valgt syv forskjellige stammer, og deres genom ble sekvensert på et Illumina Miseq-system. De sekventerte genomene ble deretter lastet opp på BAGEL4 for å lete etter bakteriocin-gener. Resultatene viste at hvert genom inneholdt putative bakteriocin-gener, og i noen tilfeller ble mer enn ett bakteriocin identifisert. I tillegg tilhørte de ulike bakteriocinene forskellige klasser, noe som illustrerer mangfoldet av bakteriociner fra melkesyrebakterier. Det siste forsøket var rensingen av det mest relevante bakteriocinet i forhold til formålet med studien. I den sammenhengen ble bakteriocinet fra *E. thailandicus* renset ved ammoniumsulfatutfelling etterfulgt av et omvendt-fase kromatografi trinn. Molekylmassen ble bestemt til å være 6312 Da ved MALDI-TOF MS, som bekrefter at det rensede bakteriocinet var den sirkulære thaiocin 1. Resultatene av studien oppfordrer bruken av thaiocin 1 fra *E. thailandicus* for å hemme veksten av *L. garvieae* i fiskeoppdrett.

Table of Contents

1. Introduction

The Plague of Justinian (541-542) and the Black Death that ravaged Europe in the fourteenth century were two devastating pandemics caused by *Yersinia pestis* (Morse, 2009) that illustrate well the morbidity of infectious diseases. To accentuate their relevance throughout history, it was reported that infectious diseases was the leading cause of death worldwide at the beginning of the 20th century (Yoneyama & Katsumata, 2006). The discovery of the first antibiotic, penicillin by Sir Alexander flaming in 1928 was therefore a major step forward in the fight against bacterial infections.

1.1 The antibiotic resistance crisis

Antibiotics are organic molecules that are naturally produced by bacteria and fungi to suppress the growth of competitive microorganisms. They are secondary metabolites made by multienzyme complexes (Latha & Dhanasekaran, 2015).

Millions of lives were saved by using penicillin to treat bacterial infections in soldiers during the Second World War (Ventola, 2015a). Since then, more than 20 new classes of antibiotics have been discovered and developed, mostly between 1930 and 1962 (Coates et al., 2011). Based on their mode of action, Kohanski et al. (2010) classified them as inhibitors of: DNA replication, RNA synthesis, protein synthesis or cell wall synthesis.

As effective as antibiotics can be, resistant pathogens always emerged. In fact, penicillin resistant *Staphylococcus aureus* were already reported by the mid-1940s (Chambers & DeLeo, 2009) and the first methicillin-resistant *S. aureus* (MRSA) were identified in the decade following the discovery of methicillin (Ventola, 2015a). By 2011, resistance has been seen to nearly all antibiotics developed whereas only five new ones were approved for therapeutic use between 2011 and 2015 to solve this problem (Ventola, 2015b). Although antibiotic resistance is an expected consequence of evolution, the antibiotic resistance crisis at the present day is a result of misuse and overuse of antibiotics. With the reduced efficiency of antibiotics, infections that were treatable in the 20th century are threatening public health now.

Infectious diseases are now the second leading cause of death worldwide and in Europe, 25 000 people are killed by drug-resistant bacteria every year (Martens & Demain, 2017). Faced with

this alarming situation, scientists are turning to alternative approaches to antibiotics for disease treatments. Bacteriocins are emerging as a promising alternative for that purpose.

1.2 Bacteriocins: A non-antibiotic approach

Bacteriocins are small antimicrobial peptides (AMPs) that are ribosomally produced by bacteria as a defense strategy. The first identified bacteriocin named colicin was discovered by André Gratia in 1925 from the Gram-negative *Escherichia coli.* Since then, a wide variety of bacteriocins has been isolated, and scientists estimated that up to 99 % of all bacteria produced at least one (Allen et al., 2014). In contrast to the broader inhibition spectrum of the antibiotics, the narrow antimicrobial spectrum of bacteriocins consists of only closely related species. However, bacteriocins from Gram-positive bacteria can inhibit bacteria from different genera and species, including several pathogens (Perez et al., 2014). The selective toxicity could be an advantage for the treatment of infectious diseases caused by antibiotic multiresistant bacteria (Martínez et al., 2016). Moreover, the risk of developing resistance is limited to the target of the narrow spectrum bacteriocin. Among the bacteriocin-producing bacteria, the lactic acid bacteria (LAB) are the best studied because most of them are commensal in the human gastrointestinal tract. Consequently, the bacteriocins they produced are not toxic to the human body as exemplified by nisin, which has been generally recognize as safe (GRAS) to be a food preservative. This property is advantageous compared to the antibiotics that have been shown to disturb the gut microbiota (Arabestani et al., 2014) . The study will therefore focus on bacteriocins produced by LAB.

LAB are Gram-positive fermentative bacteria found in the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Mokoena, 2017). In addition to producing lactic acid that inhibit foodborne pathogens in fermented food, they secrete heat stable and protease sensitive bacteriocins. The latter cause cell death by forming pores in the cell membrane of sensitive bacteria thereby leaking ions and ATP as opposed to the many different targets of antibiotics. However, the mechanism by which the pores are formed can differ among bacteriocins.

The efficacy of LAB bacteriocins against antibiotic resistant bacteria, foodborne and clinical pathogens have been confirmed by several in vitro and a few in vivo studies (Galvin et al., 1999; Mokoena, 2017; Piper et al., 2009) that will be discussed in detail later.

1.3 Classification and characterization of LAB bacteriocins

Bacteriocins were first classified into 4 groups by Klaenhammer (1993) according to their structures and characteristics (Zendo, 2011). Since then, the classification scheme has been revised by several authors including Nes et al (1996), Nes and Holo (2000), Diep and Nes (2002), Cotter et al (2005) and Nes et al (2007). The most radical modification of the original scheme suggested by Cotter et al (2005) has been generally accepted and used. The latter divided bacteriocins into 2 classes: the class I lantibiotics and the class II peptides, which contained 4 subclasses (IIa: pediocin-like, IIb: two-peptides, IIc: cyclic and IId: non-pediocin unmodified peptides) (Rea et al., 2011). However, the discovery of diverse bacteriocins in recent years requires the constant revision of the classification. To include all novel bactericin-subclasses that did not fit in the established grouping schemes, Alvarez-Sieiro et al. (2016) suggested an adjusted classification based on the biosynthesis mechanism and biological activity. This section describes the classification scheme by Alvarez-Sieiro et al. (2016) shown in figure 1.1

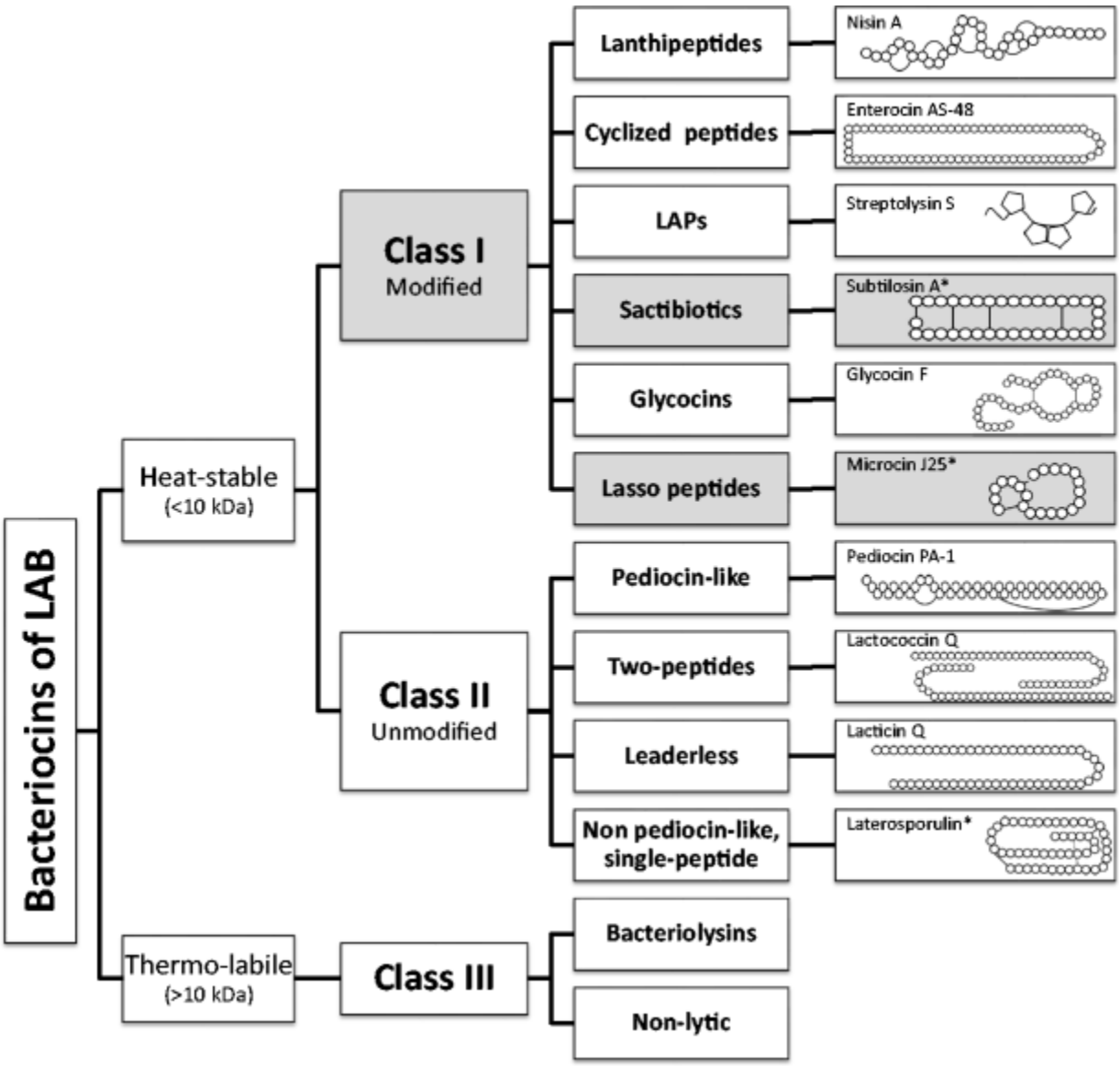


Figure 1.1 Proposed classification scheme for bacteriocins and their structures (Alvarez-Sieiro et al., 2016). The classes highlighted in gray were identified in silico and the asterix * indicates bacteriocins found in non-lactic acid bacteria. The structure of class III remains uncharacterized.

As seen in figure 1.1, the scheme is also applicable for bacteriocins from other microorganisms, but the focus of this paper is on those produced by lactic acid bacteria. Therefore, the following paragraphs describe the LAB bacteriocins in detail.

1.3.1 Class I: the ribosomally produced and post-translationally modified peptides (RiPPs)

All the peptides less than 10 kDa that undergo enzymatic modification during biosynthesis are grouped into this first class. The uncommon amino acids and structures of these modified peptide are used to further classify them as lanthipeptides, cyclized peptide, sactibiotics, linear azol(in)e-containing peptides (LAPs), glycocins or lasso peptides. The bacteriocins in this group consist of a leader peptide fused to a core peptide (Alvarez-Sieiro et al., 2016).

Class Ia: Lanthipeptides

This subclass consists of peptides that have been subjected to post-translational modification, giving rise to molecules with uncommon amino acid residues such as lanthionine or 3-methyllanthionine. The internal rings in their structure (shown in figure 1.1) are the results of covalent bonds formed by the unusual residues between amino acids (Perez et al., 2014). The lanthipeptides are further classified into four groups based on the enzyme that conduct the post-translational modification. LanBC-modified (Type I) and LanM-modified (Type II) are referred to as lantibiotics due to their antimicrobial acitivity while type III and IV have no known antimicrobial activity (Alvarez-Sieiro et al., 2016). For this reason, only the lantibiotics are further characterized.

Nisin A and its variants produced by *Lactococcus lactis* are the most extensively studied type I lantibiotics while lacticin 3147 from *L. lactis* is the best studied type II lantibiotics. Both bacteriocins create pores in the cell membrane by mechanisms involving the binding to lipid II. (Alvarez-Sieiro et al., 2016). In vitro studies showed that several lantibiotics including nisin and lacticin 3147, had a remarkable inhibitory activity against gram-positive pathogens such as *Streptococcus pneumoniae*, staphylococci (including methicillin-resistant *S. aureus* (MRSA)), vancomycin-resistant enterococci (VRE), various mycobacteria, *Propionibacterium acnes* and *Clostridium difficile* (Cotter et al., 2013). In addition, a study by Kuwano et al. (2005) demonstrated that purified nisin Z can inhibit *Escherichia coli.*

Class Ib: head-to-tail cyclized peptides

This group includes peptides with a circular backbone that resulted from the amide bond between their N- and C termini. Although they are known to be synthesized as linear peptides, the mechanism of circularization is still unclear. Due to their circular conformation, they are

structurally stable, tolerate higher temperature and pH variation, and are resistant to proteases. As an example, Sawa et al. (2009) reported that the circular lactocyclicin Q (LycQ) from *Lactococcus* sp. strain QU 12 maintained full activity after autoclaving at 121°C for 15 min at pH 3 and 4 and was highly resistant to proteases. All known circular bacteriocins can be further divided into 2 subclasses according to their biochemical characteristics and their sequence similarity. Group (i) represented by the model bacteriocin enterocin AS-48 consists of the cationic peptides with high isoelectric point (pI ~ 10) while group (ii) includes peptides with low isoelectric point (pI~5) and high sequence similarity (van Belkum et al., 2011). Despite sharing similar physicochemical features, the bacteriocins within the same subgroup such as carnocyclin A and enterocin AS-48 can differ in their mode of action. One of the differences reported lies in the pores created, which are anion selective in the case of carnocyclin A and non-selective for enterocin AS-48 (Gong et al., 2009). Generally, all the circular bacteriocins have a broad antimicrobial activity spectra and are especially effective against foodborne pathogens within the *Listeria* and *Clostridium* genera (Gabrielsen et al., 2014).

Class Ic: sactibiotics

Sactibiotics have cross-linkages between the sulphur of cysteine residues and the alpha carbon of any other amino acid residues of the same peptide as a result of post translational modification. Although no studies reporting sactibiotics isolated from lactic acid bacteria have been found at the time of the writing, Alvarez-Sieiro et al. (2016) reported a putative sactibiotic gene in silico in the genera *Enterococcus*, *Lactococcus* and *Streptococcus*. The most studied sactibiotic is the circular subtilosin A produced by the non-lactic acid bacterium *Bacillus subtilis*, which differ from the circular LAB bacteriocins in class Ib in its smaller size and its extensive post translational modifications (Rea et al., 2011).

Class Id: linear azol(in)e-containing peptides (LAPs)

LAPs are linear peptides characterized by the heterocyclic amino acids oxazoles and thiazoles that were obtained from the modification of cysteine, serine and threonine residues by cyclodehydration and dehydrogenation reactions (Alvarez-Sieiro et al., 2016). This group includes the pore forming toxin, streptolysin S produced by nearly all *Streptococcus pyogenes* (Molloy et al., 2011). Although the mechanism of pore formation is still unclear, streptolysin S

have been reported to lyse erythrocytes (red blood cells), lymphocytes, neutrophils, platelets, subcellular organelles (Barnett et al., 2015), and wall less bacteria (Bernheimer, 1966).

Class Ie: glycocins

This group consist of peptides to which sugar molecules (moieties) are attached through a post translational modification called glycosylation. The first glycocin described in lactic acid bacteria was glycocin F produced by *Lactobacillus plantarum* KW30 (Stepper et al., 2011). In their paper, Stepper et al. (2011) showed that glycocin F contained two disulfide bonds and two N-acetylglucosamine moieties (GlcNAc). One of them is the result of an extremely rare post translationally modification in which GlcNAc is linked to cysteine while the other one is the more common linkage of GlcNAc to serine. Even though the mechanism of action of glycocin F is still unclear, its bacteriostatic activity has been reported against strains in the genera *Streptococcus*, *Enterococcus* and *Bacillus* (Kerr, 2013).

Class If: lasso peptides

Lasso peptides are characterized by their 3D structure resembling the lasso of a cowboy. The structure results from the bond between the N-terminal amine and a negatively charged residue in the core region (Alvarez-Sieiro et al., 2016). No lasso peptides from LAB have been reported at the time of the writing, but Alvarez-Sieiro et al. found putative lasso peptides in streptococci. Microcin J25 produced by *E. coli* (Salomon & Farias, 1992) is a member of this class.

1.3.2 Class II: the unmodified bacteriocins

All bacteriocins less than 10 kDa that contain standard amino acid residues can be grouped in this class. Apart from the involvement of a leader peptidase and/or a transporter, their maturation does not require additional modification enzymes (Alvarez-Sieiro et al., 2016). Bacteriocins in this group are further divided into 4 subclasses: pediocin-like, two-peptides, leaderless and non-pediocin-like single peptides.

Class IIa: pediocin-like bacteriocins

The bacteriocins in this group are referred to as pediocin-like after the first characterized member pediocin PA-1 produced by *Pediococcus acidilactici* strain PAC-1.0 (Henderson et al., 1992). Pediocin-like bacteriocins are characterized by a conserved sequence (YGNGVXC) and one or more disulfide bridges at their N-terminal region. The conserved sequence is responsible for their

remarkable effectiveness against *Listeria monocytogenes* while the disulfide bridges intensify their antimicrobial activity (Perez et al., 2014). In fact, the stronger antimicrobial activity of enterocin NKR-5-3C against *L. monocytogenes* is attributed to its two disulfide bridges (Himeno et al., 2012). Diep et al. (2007) reported that pediocin-like bacteriocins lyse other bacteria by binding to the sugar transporter mannose phosphotransferase system (Man-PTS) receptors, which is followed by their insertion into the cytoplasmic membrane thereby forming pores.

Class IIb: two-peptide bacteriocins

Two-peptide bacteriocins consist of two different peptides that only manifest their highest antimicrobial activity when present in equal amounts. The presence of both peptides is required for bacteriocin activity in some cases such as lactococcin G or lactococcin Q produced by *Lactococcus lactis* (Nissen-Meyer et al., 1992; Zendo et al., 2006). Both lactococcin G and lactococcin Q only inhibit strains of *L. lactis.* On the other hand, some two-peptide bacteriocins such as thermophilin 13 from *Streptococcus thermophilus* (Marciset et al. 1997), show bacteriocin activity as separate peptides. Marciset et al. (1997) also reported that thermophilin 13 inhibited *S. thermophilus, C. botulinum, L. monocytogenes,* and *B. cereus* in their study. Studies of several two-peptide bacteriocins revealed that they created sophisticated pores in the cell membrane (Nissen-Meyer et al., 2010). The type of small molecules they allow across the membrane is however specific to each two-peptide bacteriocin.

Class IIc: leaderless bacteriocins

Unlike the typical bacteriocins synthesized with a N-terminal leader peptide attached to a C-terminal propetide, the members of class IIc are produced as leaderless bacteriocins. Another biosynthetic feature of this group is the lack of genes encoding immunity proteins (Alvarez-Sieiro et al., 2016). Enterocin L50 produced by *Enterococcus faecium* L50 (Cintas et al., 1998) is a two-peptide bacteriocin that has been classified as a leaderless bacteriocin due to some differences from other two-peptide bacteriocins. Unlike the latter, the two peptides of enterocin L50 (L50A and L50B) are very similar and exhibit significant antimicrobial activity separately (Nes et al., 2001).

Among the leaderless bacteriocins, the mechanism of action of lacticin Q produced by *L. lactis* (Fujita et al., 2007) has been extensively studied. The cationic lacticin Q does not interact with a specific receptor but binds to the negatively charged membrane to form a huge toroidal (ring-

shaped) pore. Although no cell receptor is involved, the killing mechanism is selective against Gram-positive bacteria and not Gram-negative bacteria (Perez et al., 2014). Fujita et al. (2007) reported that lacticin Q had an antibacterial spectrum as broad as that of nisin A and it inhibited bacteria in the genus *Bacillus, Enterococcus, Lactococcus* and *Staphylococcus.*

Class IId: non-pediocin-like, single-peptide bacteriocins

This group consists of the remaining unmodified bacteriocins that do not fulfill the criteria of the other classes. They are unrelated, linear, one-peptide bacteriocins that have different structures, mechanisms of action and secretion (Alvarez-Sieiro et al., 2016).

The first isolated member of this group, lactococcin A from *L. lactis* subsp. *cremoris.* (Holo et al., 1991) could only inhibit strains in the *Lactococcus* genus and had no apparent sequence similarity to other known bacteriocins. Although structurally different from the pediocin-like bacteriocins (class IIa), lactococcin A also bind to the man-PTS to cause membrane leakage. However, lactococcin A only recognize lactococcal man-PTS and binds different regions of the receptor than the pediocin-like bacteriocins (Kjos et al., 2011).

Lactococcin 972 from *L. lactis* (Martinez et al., 1999) is another member of this class that only inhibit strains in the genus *Lactococcus.* Unlike lactococcin A, it stops cell wall synthesis by binding to the cell wall precursor lipid II (Martinez et al., 2000).

Laterosporulin shown as an example of class IId in figure 1.1 is produced by the non-lactic acid bacterium *Brevibacillus* sp. (Singh et al., 2012) and was reported to inhibit both Gram-negative and Gram-positive bacteria.

1.3.3 Class III

Class III bacteriocins are heat-labile proteins larger than 10 kDa that are composed of different domains (Alvarez-Sieiro et al., 2016). The bacteriocins in this class can be further divided into 2 subclasses based on their mode of action: bacteriolytic and non-lytic. Enterolysin A produced by *Enterococcus faecalis* (Nilsen et al., 2003) is a bacteriolysin that is composed of an N-terminal catalytic domain and a C-terminal substrate recognition domain. It hydrolyzes the peptide bonds in the peptidoglycan of strains in the genus *Lactobacillus, Lactococcus, Pediococcus, Enterococcus, Listeria, Bacillus, Staphylococcus, Propionibacterium* (Nilsen et al., 2003).

On the other hand, dysgalacticin produced by *Streptococcus dysgalactiae* is a non-lytic bacteriocin that cause cell death by inhibiting the sugar uptake and by disrupting the membrane permeability of sensitive *Strep. pyogenes* (Swe et al., 2009).

1.4 Biosynthesis of LAB bacteriocins

The genes that are involved in the biosynthesis of LAB bacteriocins are often clustered together on chromosomes, plasmids and/or transposons (Perez et al., 2014). In general, the bacteriocin gene cluster essentially consists of a structural gene, an immunity gene and a transporter gene as shown in figure 1.2. Other genes related to biosynthesis and maturation are present in the cluster for class I bacteriocin. Sometimes, the transport proteins are also involved in immunity especially for some class II bacteriocins (Kjos et al., 2011) and some circular bacteriocins (Class Ib).

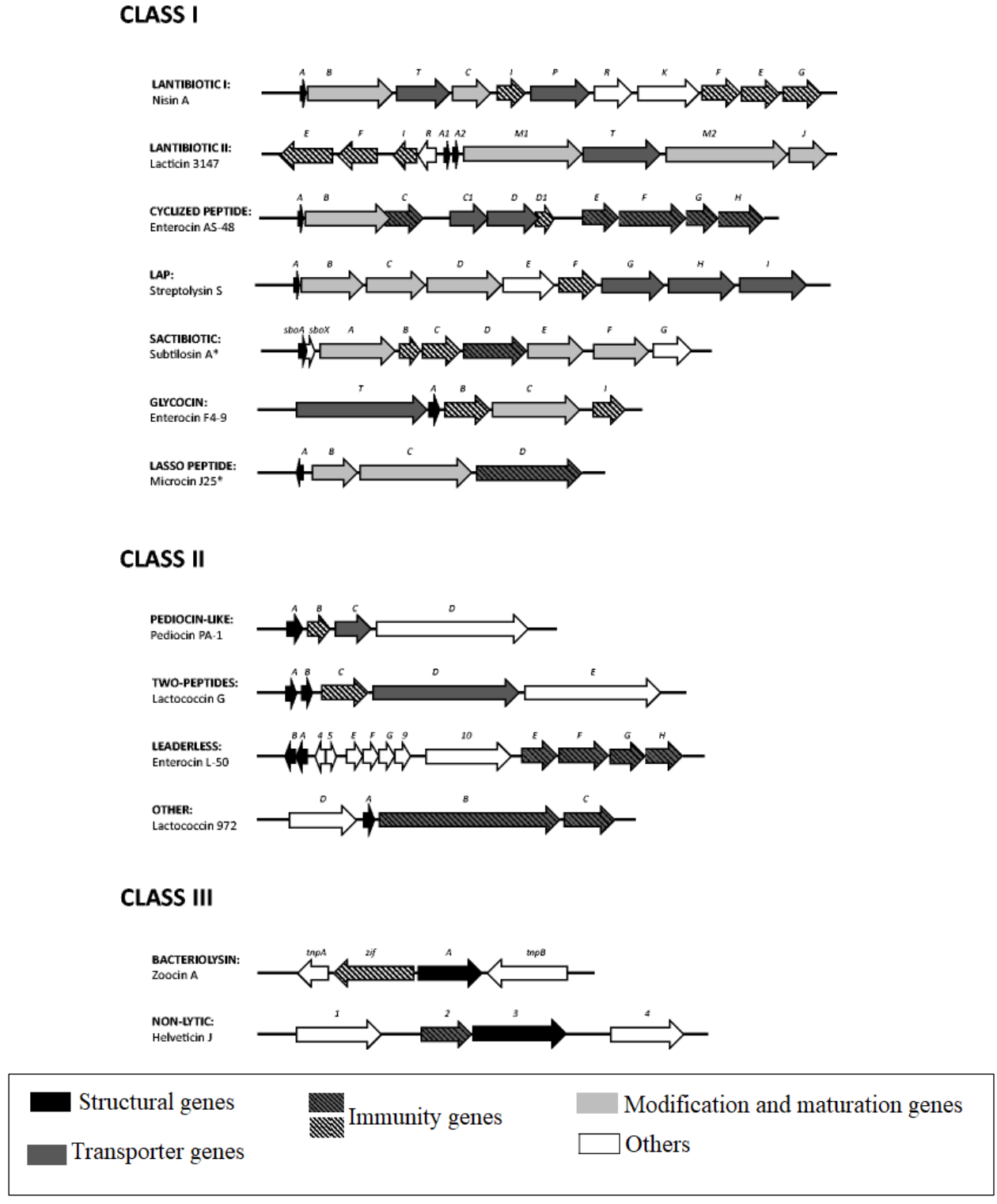


Figure 1.2. Schematic representation of bacteriocin gene clusters (not drawn to scale) adapted from Alvarez-Sieiro et al. (2016). *Bacteriocins from non-lactic acid bacteria

For the modified bacteriocins in class I, the peptide synthesized from the structural gene is inactive and is made up of an N-terminal leader sequence attached to the C-terminal propeptide. The different functions of the leader peptide are (i) to act as the recognition site of enzyme for maturation and transport proteins, (ii) to keep the bacteriocin inactive inside the cell thereby protecting the producer and (iii) to ensure a suitable conformation between the propeptide and the biosynthetic enzymes during maturation (Perez et al., 2014). Maturation involves enzymatic processes that differ among bacteriocins in class I (Riips) and the removal of the leader peptide during the transport outside of the cell. Immunity proteins and/or specialized ABC transporter system protect the producer against its own bacteriocin by interacting with the latter.

Most of the unmodified bacteriocins in class II are also synthesized with a leader peptide except for the leaderless bacteriocins of class IIc that are produced as active bacteriocins. The biosynthesis of the latter remains unclear. For the other unmodified bacteriocins, the leader peptide is cleaved off by specific enzymes as they are transported to the extracellular space through an ABC transporter, sometimes associated with an accessory protein (Perez et al., 2014). The immunity proteins secreted by class II bacteriocins are very diverse and the exact mechanism behind immunity is still unclear. However, Morten et al (2011) reported that the immunity proteins of lactococcin A and pediocin-like bacteriocins lock the latter on the Man PTS receptor to prevent pore formation.

The biosynthesis mechanism of class III bacteriocins is the least characterized among the three classes. A study conducted by Malinicova et al (2011) on enterolysin A revealed that a gene encoding for an endopeptidase C39, which is involved in bacteriocin maturation and secretion, was found in the region downstream of the enterolysin A gene. However, they did not detect any genes similar to known bacteriocin immunity proteins nearby. On the other hand, an immunity gene (*zif* gene) was found close to the gene encoding for the bacteriolysin zoocin A (Alvarez-Sieiro et al., 2016).

1.5 Production and regulation of LAB bacteriocins

Several studies have reported that the production of LAB bacteriocins can be influenced by pH, temperature, media composition, type of media (solid or liquid), incubation time, the producing strain and salt concentration (Neysens et al., 2003; Nilsen et al., 2003; Turgis et al., 2016; Yang,

1994). However, the regulation of bacteriocin production is usually controlled by signal molecules called induction factors through a quorum sensing system. The latter is a regulatory system in which bacteria use signal molecules to sense the cell density in the surrounding and then adjust their gene expression accordingly. A high concentration of the induction factor in the surrounding implies that there are sufficient bacteriocin producers to inhibit the growth of the competitor (Bemena et al., 2014). In such a situation, the induction factor is allowed to bind to a specific receptor called histidine protein kinase on the cell surface. The binding activates the protein receptor, which phosphorylates the response regulator. The latter then initiates the transcription of the bacteriocin gene cluster by binding to the promoter region. While several bacteria that produce class II bacteriocins (for example sakasin P) use a dedicated peptide pheromone as the induction factor, the lantibiotics (nisin, subtilin) use their own bacteriocin (Dimov et al., 2005).

Studies have shown that LAB strains in the genera *Lactobacillus, Enterococcus* and *Leuconostoc* can produce several bacteriocins (Ishibashi et al., 2014). The secreted bacteriocins can be similar or can belong to different classes. Ishibashi et al. (2012) reported that *E. faecium* NKR-5-3 can produce 4 different enterocins (NKR) simultaneously: the one component NKR-5-3A from the two-peptides NKR-5-3A and NKR-5-3Z, the circular NKR-5-3B (Perez et al., 2016), the pediocin-like NKR-5-3C, and the non-pediocin linear NKR-5-3D. Although the knowledge about the regulation of simultaneously produced bateriocins are still limited, Ishibashi et al. (2014) identified a wide-range ABC transporter that secrete Ent53A/Ent53Z, Ent53C and Ent53D. In the case of *Carnobacterium piscicola* LV17, the production of carnobacteriocins B2, BM1 and A is controlled by 2 regulatory systems: one controlling the production of the pediocin-like B2 and BM1, and the other regulating the non-pediocin like A (Eijsink et al., 2002).

1.6 Potential applications of LAB bacteriocins

The characteristics that distinguish LAB bacteriocins from antibiotics can be exploited in various fields including in food industry, in animal and human medicine, and in aquaculture.

In the food industry, some LAB bacteriocins are used as biopreservatives due to their non-toxicity to humans, their stable structure and their ability to inhibit spoilage bacteria such as *L. monocytogenes, S. aureus, Bacillus cereus, Clostridium botulinum* and *Clostridium tyrobutyricum* (Martínez et al., 2016). As an example, the lantibiotic nisin has been approved as

GRAS worldwide and is now used as food preservative in many products in more than 60 countries (López-Cuellar et al., 2016). In some countries, pediocin PA-1 (clas IIa) is also commercially available and is used to inhibit *L. monocytogenes* in meat products (Yang et al., 2014). To control *Listeria* growth in ready-to-eat food, the use of *Carnobacterium maltaromaticum*, which produces the circular carnocyclin A has been legalized in Canada, the United States, Mexico, Costa Rica and Columbia (López-Cuellar et al., 2016). There are several studies that demonstrate the potential of other LAB bacteriocins in the food industry, but regulatory authorities must approve them before further action can be taken.

Although LAB bacteriocins have a narrow inhibition spectrum compared to antibiotics, some of them can inhibit Gram-positive human and animal pathogens. The mentioned ability and the reduced risk of resistance associated with their selective toxicity are advantageous in the field of medicine.

The ability of the lantibiotics (class Ia) to inhibit Gram-positive human pathogens is well documented. In fact, several in vitro studies showed that lacticin 3147 and nisin are effective against methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE), *Streptococcus pneumonieae* and *Clostridium difficile* (Galvin et al., 1999; Piper et al., 2009). In addition, in vivo studies conducted on mice by Mota-Meira et al. (2005) showed that mutacin B-Ny266 was active against MRSA. The combination of nisin with cell wall inhibitors (antibiotics) were also found to be an effective way to kill antibiotic resistant bacteria (Brumfitt et al., 2002). On the other hand, Mokoena (2017) reported that bacteriocin-producing strains of *Lactobacillus* could inhibit uropathogens including the Gram-negative *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.*

Studies on dairy animals suggest the use of the lantibiotics nisin, lacticin 3141 and macedocin ST91KM to treat the most economically costly disease, mastitis (López-Cuellar et al., 2016). In poultry, the growth of *Salmonella* species have been controlled by divercin AS7 (class IIa) produced by *Carnobacterium divergens*. (Bemena et al., 2014).

Aquaculture is an important food-producing sector that also faces the antibiotic resistance crisis. In order to limit the use of the latter in aquatic environment, LAB probiotics have been tested, mostly in fish farming. A study conducted by Robertson et al. (2000) showed that administrating *Carnobactium* sp. from salmon bowel to the feed of rainbow trout and Atlantic salmon

contributed to their resistance against several known fish pathogens. The effect of using LAB probiotics intended for human and animals in fish was investigated by Nikoskelainen et al. (2001). They reported that *Lactobacillus rhamnosus* and *Lactobacillus bulgaricus* were the best probiotic candidates for rainbow trout. Even though probiotics showed positive results in these studies, the duration of treatment should be further investigated.

LAB bacteriocins clearly have a potential in the food industry, human and animal medicines and aquaculture. However, more in vivo analyses with animal models and clinical trials should be conducted before their practical application.

1.7 The relevance of *Lactococcus garvieae* in the study

The purpose of this study was to isolate and characterize bacteriocins produced by lactic acid bacteria that could be used against *L. garvieae* B1678, isolated from trout.

L. garvieae is a lactic acid bacterium that was first isolated from bovine mastitis (Collins et al., 1983) but is mostly known as a fish pathogen. The bacterium causes a hyperacute hemorrhagic septicemia called lactococcosis in many fish species including rainbow trout, grey mullet and Japanese yellowtail. The economic losses associated with farmed rainbow trout are particularly high, with a reported mortality rate of 80 % (Vendrell et al., 2006). Aside from the mentioned hosts, *L. garvieae* has also been identified in porcine blood, poultry meat, milk, dairy products, vegetable and humans. Although it is rarely pathogenic to humans, a few cases of infective endocarditis, liver abscess, diverticulitis, peritonitis, endophthalmitis and spondylodiscitis have been reported (Eraclio et al., 2018). In most of these cases, the patients had underlying gastrointestinal diseases and the consumption of raw fish was noted. *L. garvieae* is therefore an emerging opportunistic pathogen in humans.

The precise virulent factors involved in the pathogenicity of *L. garvieae* are still unknown. However, the latest study on the matter suggested that the genes encoding for hemolysin, fibronectin-binding protein and penicillin acylase were essential for the virulence of the bacterium (Eraclio et al., 2018).

L. garvieae is a multiple bacteriocin producer. To date, it has been reported to secrete 5 bacteriocins: the least characterized garviecin L1-5, the class IId garvieacin Q, the class IId garvicin A, the circular garvicin ML and the leaderless garvicin KS.

2. Materials and Methods

2.1 The origin of the samples

In this study, 50 samples of fermented fruits and vegetables from the frozen stocks of LMG (Laboratory of Microbial Gene Technology) were used as a source for lactic acid bacteria. The fruits and vegetables were previously bought at a Turkish store in Hauketo (Oslo) and prepared separately in a normal kitchen. To create different environments, each sample was fermented in two separate tubes: one with only tap water, and the other tube with tap water and about 0.5 % sodium chloride. After three weeks of fermentation in an outdoor storage room, 15 % glycerol stocks of the samples were made and stored at - 80 ºC.

The sources of the 50 samples are listed in the Appendix Table 1.

2.2 Preparation of the culture media

The choice of the culture media depended on the bacteria involved. However, three different forms of the media were made throughout the study: solid agar, soft agar and broth. To prepare the growth medium, the amount recommended by the manufacturer was first weighed, then added to a bottle filled with MilliQ water, which was purified by a Millipore E-pod ^{TM} (Elix). In the next step, the medium powder was dissolved completely by using a magnetic stir bar and a magnetic stirrer MR 3001 (Heidolph). Depending on the form needed, agar powder was added: 15 g/L to make solid agar, 8 g/L to make soft agar and no agar powder for the broth. Finally, the bottles were autoclaved at 121°C for 15 minutes. The following paragraphs describe the different culture media used in this study.

de Man, Rogosa and Sharpe (MRS, Oxoid) is a medium designed to meet the nutritional need of lactic acid bacteria, especially *Lactobacillus* species. It contains sodium acetate that inhibits the growth of other competing bacteria such as streptococci, and moulds. The concentration indicated by the manufacturer is 52 g/L.

Brain-Heart infusion (BHI, Oxoid) is a nutrient-rich medium that support the growth of nutritionally demanding bacteria, including many pathogens. The concentration recommended by the manufacturer is 37 g/L.

GM17 was prepared from the medium M17 (Oxoid) by dissolving 37.25 g in 950 ml of MilliQ water and by adding 10 ml of a 40 % glucose solution. The high concentration of glucose provides a faster growth of lactic acid bacteria.

Todd Hewitt (TH, Oxoid) is a growth medium used to cultivate streptococci that are particularly demanding in terms of nutrition and environment such as the fastidious *Streptococcus pneumoniae.* To prepare this medium, 36.4 g of TH powder was dissolved in 1 liter of MilliQ water.

2.3 The screening assay

2.3.1 The first round of screening

The following screening procedure is an established sandwich overlay method used at LMG that consists of adding several layers of medium to an agar plate. In preparation for the screening, 10 µl of the frozen sample was diluted in 1 ml of 0.9 % NaCl. A series of three dilutions were then prepared from this original sample using MRS soft agar kept at 48 ºC on a water bath. A pipetboy (Integra) was used when handling warm agar because it tolerates higher temperatures, thus providing accurate dosing. It is worth mentioning that the following dilutions were chosen because a former master student at LMG observed sufficient colonies during trials with the same samples (Haldorsen, 2017).

To make the first dilution, 10 µl of the previously diluted original sample was vigorously mixed with 5 ml of MRS soft agar in a test tube. The second dilution was made by taking 50 µl from dilution 1 to another tube filled with 5 ml of MRS soft agar. After vortexing dilution 2, 50 µl was transferred to the last tube containing 5 ml of MRS soft agar to prepare dilution 3. Each dilution was then spread plated into three separate MRS agar plates. When the agar had solidified, 5 ml of MRS soft agar was added on top of it. This middle layer would prevent the mixing of the sample and the indicator layer when the latter would be added. The dry agar plates were then placed in a jar with AnaeroGen 3.5 L Sachets (Thermo Scientific™ Oxoid) for an overnight (ON) anaerobic incubation at 30 ºC. As a preparation for the next day, an ON culture of *L. garvieae* B1678 was made by inoculating a 5 ml MRS broth with bacterial cells scraped by a toothpick from the frozen stock. This indicator was also incubated at 30 ºC. On the second day, the fresh culture of *L. garvieae* B1678 was mixed with MRS soft agar in the proportion: 500 µl

bacterial culture per 100 ml MRS agar. Then, 5 ml of the indicator was distributed to the plates before they were incubated aerobically at 30 ºC. The plates with colonies showing clear inhibition zones against *L. garvieae* on the third day were picked for further work.

During the first trials, a fourth layer of MRS soft agar mixed with the nisin Z producer *L. lactis* strain B1627 was added to the plates that showed inhibition zones on the third day. The purpose of this last layer was to exclude bacteria that produce the well-known bacteriocin nisin. Assuming that nisin producers would be immune to their own bacteriocin, the growth of *L. lactis* B1627 on top of the inhibition zones seen on day 3 would indicate that the antimicrobial was nisin or a similar bacteriocin. However, the results observed on day 4 were not reliable because the *L. lactis* layer was not clearly distinguishable from the third layer. As the inhibition zones were still present after this fourth layer, it was easy to mistakenly assume that the bacteria from the sample produced something different from nisin. Another problem was that this additional layer could contaminate the colonies that needed to be picked later. Consequently, the fourth layer was discarded and the *L. lactis* B1627 was used in a second round of screening instead. An illustration of the screening technique described in this section is shown in figure 2.1.

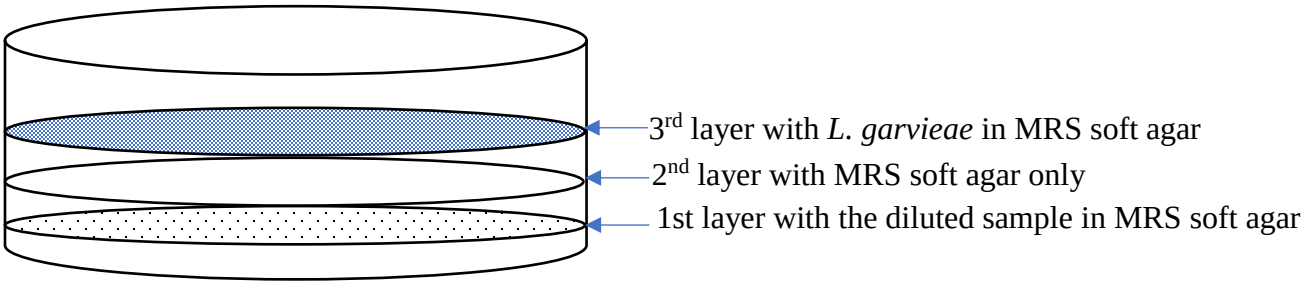


Figure 2.1. Schematic representation of the screening technique. The figure represents one MRS agar plate with the three different layers of media.

2.3.2 The second round of screening

The colonies that produced inhibition zones after the ON incubation with the indicator layer (3rd layer) were picked to obtain pure cultures. A sterile toothpick was used to stab the colony of interest and to streak it out on an MRS agar plate. After an ON incubation at 30 ºC, single colonies from the plate were isolated again and re-streaked on a new MRS agar plate. The latter was incubated ON and was then kept at 4 ºC as they became the main source of the pure cultures. A second round of screening was conducted on the pure cultures to confirm that they still inhibit *L. garvieae* B1678 like on the first round of screening. In addition, they were also tested against the nisin Z producer *L. lactis* B1627 to check if the produced antimicrobial substances were nisin. To prepare for the second screening, ON cultures of these two indicators were made from frozen stock using MRS broth. The following day, 25 µl of each indicator was mixed with 5 ml of MRS soft agar and plated as lawn on separate MRS plates. Pure cultures were then streaked on the plated MRS agar before an ON incubation at 30 ºC. The colonies that produced clear inhibition zones were picked with a toothpick and transferred to a 5 ml MRS broth for ON incubation. Finally, 15 % glycerol stocks of the pure cultures were made by mixing 0.5 ml of a 45 % glycerol solution with 1 ml of the ON culture. They were stored at -80 ºC until further use.

2.4 Identification of the isolated bacteria

In order to identify the isolated pure cultures, DNA technologies including 16S ribosomal RNA (rRNA) sequencing and repetitive extragenic palindromic (REP) PCR profiling were utilized. A common DNA isolation step was required prior to the DNA analyses.

2.4.1 Extraction of genomic DNA using glass beads and miniprep-columns

The choice of a DNA extraction method usually depends on the nature of the starting sample and the final application. A protocol adapted for the isolation of DNA from LAB bacteria was followed by using the E.Z.N.A ® plasmid DNA minikit I (Omega Bio-tek). The latter could be used to isolate genomic DNA because a bead-beating step to generate smaller DNA fragments was included in the protocol. Since the identity of the bacteria was unknown and the downstream application involves PCR reactions, glass bead beading was also a suitable cell lysis method.
To harvest the bacterial cells, 4.5 ml of ON culture in MRS broth were spun down at 13 000 x g (maximum speed) for one minute. After discarding the supernatant, the cell pellet was washed in 200 µl of TBS-buffer at pH 7.4 and centrifuged at maximum speed for one minute. The

supernatant was removed before the pellet was resuspended in 350 µl of cold solution I, which contained tris(hydroxymethyl) aminomethane (Tris), glucose, ethylenediaminetetraacetic acid (EDTA) and RNase A (Oswald, 2014). While Tris provides an optimal pH (8) for cell lysis, the glucose maintains the pH in the range 12-12.5 that is essential for denaturation at a later step (Birnboim & Doly, 1979). On the other hand, EDTA inactivates DNases and weakens the bacterial cell wall whereas RNase A degrades cellular RNA (Oswald, 2014).
For the next step, the cell suspension was transferred to a FastPrep tube with 0.5 g of acid-washed glass beads (≤ 106 µm, Sigma -Aldrich). The latter was then placed in the FASTprep ® 24 machine (MP biomedicals) set to run 3 times for 20 seconds at the speed of 4 m/s. After lysing the cells and shearing the DNA into small pieces in the process, the fast-prep tube was centrifuged for 5 seconds to separate the glass beads from the mixture. The liquid phase was transferred to a new Eppendorf tube before adding 250 µl of solution II. The latter consisted of NaOH that denatures DNA and sodium dodecyl sulfate (SDS), which dissolves cell membranes and denatures proteins (Oswald, 2014). The next step was the addition of 350 µl of solution III, which contained potassium acetate that renature small DNA fragments and precipitates SDS (Dr.Biology, 2010). Next, the insoluble proteins and the SDS were separated from the soluble renatured DNA fragments by a 10-minute centrifugation at maximum speed. The cleared supernatant was later transferred to a HiBind DNA Mini Column placed in a collection tube and centrifuged at maximum speed for one minute. During this step, the negatively charged DNA bound to the silica glass fiber column while the contaminants were removed in the flow-through. The column was then washed with 750 µl of DNA wash buffer diluted with 90 % ethanol to remove any remaining contaminants. Two rounds of centrifugation at maximum speed for one minute, with the removal of the flow-through in between, was then conducted. Finally, the column was placed in a new Eppendorf tube and the genomic DNA was eluted with 40 µl of sterile water. One last centrifugation at maximum speed for one minute ensured that all the genomic DNA was in the eluate.

To check the success of the extraction process, the DNA concentration of the eluate was measured by a nanodrop ND-1000 (NanoDrop Technologies). First, the nucleic acid program was selected, and sterile water was used to blank the instrument. Then, 2 µl of the eluted DNA was loaded onto the pedestal of the instrument before the absorbances at 260 nm and 280 nm were measured. The nanodrop displayed the concentration in ng/µl and a ratio for A260/A280

that indicated potential protein contamination of the DNA sample. Pure DNA should have a A260/A280 around 1.8 whereas a lower ratio indicates protein contamination.

2.4.2 16S rRNA gene amplification and sequencing

The 16S rRNA gene is a DNA region of about 1550 bp found in all prokaryotes, that can be used to identify and classify them. The gene consists of hypervariable regions flanked on both sides by highly conserved regions, which make it possible to construct universal primers (Clarridge, 2004). Sequencing the hypervariable regions provide a signature sequence belonging to a specific species. Prior to sequencing, the extracted genomic DNA from 2.4.1 was prepared by amplifying the 16S rRNA gene through a PCR reaction using OneTaq® DNA polymerase (New England Biolabs). The universal primer pair 11F (5'-TAA CAC ATG CAA GTC GAA CG-3') and 4R (5'-ACG GGC GGT GTG TRC-3') were chosen because they usually generate sequences of about 1000 bp, which provide a correct identification. However, the mentioned pair did not always result in successful amplification of the samples, therefore the second pair 11F and 5R (5'-GGT TAC CTT GTT ACG ACT T-3') were also used.

A desired volume of master mix was made in an Eppendorf tube by mixing the PCR components shown in table 2.1. Then, 48 µl of the master mix was distributed to PCR strips before adding 2 µl of the genomic DNA as template. The PCR strips were finally placed in a S1000™ thermal cycler (Bio-Rad), which was run according to the program in table 2.2

Table 2.1 The Master mix composition for the 16S PCR

Components	**For a 50 µl reaction**	**Final concentration**
5x OneTaq Standard reaction buffer	10 µl	1X
10 mM dNTPs	1 µl	200 µM
10 µM Forward Primer	1 µl	0.2 µM
10 µM Reverse Primer	1 µl	0.2 µM
OneTaq DNA polymerase	0.25 µl	1.25 units/50 µl PCR
dH_2O	34.75 µl	--

Table 2.2 The thermocycling conditions used for the 16S rRNA PCR

Procedure	Temperature	Reaction time	Cycles	Comments
Initial denaturation	94 ºC	5 min	1	The high temperature triggers the denaturation of the double stranded DNA template.
Denaturation	94 ºC	45 sec	30	This step keeps double stranded DNA denatured.
Primer annealing	58 ºC	1 min		The temperature, which is 5 ºC below the melting temperature of the primer pair promotes the binding of the latter to the template.
Primer extension	72 ºC	1.5 min		The temperature is increased to the DNA polymerase's optimum range so that it can synthesize and elongate new DNA strands.
Final extension	72 ºC	5 min	1	The incomplete DNA ends are filled and DNA are allowed to reanneal.
Hold	4 ºC	-	-	Storage of the PCR products

After the PCR reaction, the size of the PCR products was visualized by gel electrophoresis. The gel was made up of 1 % Ultrapure ™ Agarose (Invitrogen) in TAE buffer with added peqGREEN (2 µl/50 ml). In a PCR strip, 5 µl of each PCR product was mixed with 1 µl of 6 X loading dye that contained xylene and boron monofluoride. The sample mixtures and 10 µl of a 1 kb DNA ladder were then applied to the wells of the gel before the electrophoresis system (Bio-Rad) was run at 100 V for 30 minutes. The gel was finally visualized on a molecular Imager ® Gel doc ™ XR + System (Bio-rad).

The PCR products showing a clear band around 1000 bp in the gel electrophoresis were next purified using the NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel) to remove impurities such as primer dimers, nucleotides and enzymes. In the first step, 50 µl of the PCR

products was mixed with 100 µl of the binding buffer NTI. The mixture was then applied onto the provided column placed in a collection tube and centrifuged for 30 seconds at 10 000 x g. After discarding the flow through, the DNA bound to the silica membrane in the column was washed twice with 700 µl of Buffer NT3 (with added ethanol). To remove any remaining NT3, the column was centrifugation for one minute. Finally, the DNA was eluted with 30 µl of Buffer NE (5 mM Tris/HCL, pH 8.5)

A nanodrop measurement as described in 2.4.1 was performed to determine the DNA concentration, which was then adjusted to 20-80 ng/µl with sterile water. In an Eppendorf tube, 5 µl of the diluted sample was mixed with 5 µl of one of the primers used in the PCR but diluted to 5 µM. The samples were then sent to GATC Biotech (Germany) for sequencing.

2.4.3 Repetitive extragenic palindromic (REP) PCR profiling

REP PCR is a DNA fingerprinting technique that enables the distinction between bacterial strains based on their unique profile. The method relies on the presence of repetitive and highly conserved nucleotide sequences of 21-65 bases found in the extragenic space of the bacterial genome (Tobes & Pareja, 2006). Specific primer pairs complementary to the repeated sequences are used to amplify the DNA between consecutive repetitive elements by PCR, which generates DNA fragments of different sizes (Versalovic et al., 1994). Since bacterial strains vary in the distances between their repetitive sequences, the genomic fingerprints visualized on a gel electrophoresis are specific to a strain.

A REP PCR master mix was prepared according to Table 2.3. The primer pair used: Rep-1R (5'-IIIICGICGICATCIGGC-3') and Rep-2I (5'-ICGICTTATCIGGCCTAC-3') contained inosine, which can base-pair with any natural nucleotide. The mentioned property reduces template-primer mismatches, thus making inosine-containing primers particularly useful during the amplification of similar genes. After the desired volume of PCR master mix was made in an Eppendorf tube, 20 µl was distributed to the wells of PCR strips. Next, the extracted genomic DNA from 2.4.1 was normalized to 50 ng/µl before transferring 2 µl as DNA template to the PCR strips. The volume in each well was then filled to 25 µl with distilled water. Finally, the strips were placed in the S1000^{TM} thermal cycler (Bio-Rad) that was run according to the program in table 2.4.

Table 2.3 The composition of the Rep-PCR master mix

Components	**Volume for 2 PCR reactions (in µl)**
5x OneTaq standard buffer	10
10 mM dNTPs	1
10 µM REP-1R Primer	5
10 µM REP-2I primer	5
OneTaq DNA polymerase	0.5
dH_2O	18.5
Total	40

Table 2.4 REP PCR program

Procedure	**Temperature**	**Reaction time**	**Cycles**
Initial denaturation	95 ºC	7 min	1
Denaturation	94 ºC	1 min	30
Primer annealing	41 ºC	1 min	
Primer extension	65 ºC	3 min	
Final extension	65 ºC	16 min	1
Hold	4 ºC	-	-

After the PCR reaction, 10 µl of the products mixed with 1 µl of loading dye, and 1 kb DNA ladder were applied on 1.7 % agarose (Ultrapure ™ Agarose, Invitrogen) gel. The electrophoresis system was then run at 80 V and 80 Amp current. After 3 hours, the DNA fingerprints were visualized on a molecular Imager ® Gel doc ™ XR + System (Bio-rad).

2.5 Antimicrobial spectrum of the identified bacteria

Before the inhibition spectrum of the bacteria was determined, their ability to produce the antimicrobial substances was first assessed on MRS and BHI agar plates. The spot-on-lawn method was used for this purpose and for the inhibition test. The technique consists of plating the indicator layer as a "lawn" and spotting the bacteria of interest on top of it. The medium plate that promoted the most antimicrobial production was then chosen for the antimicrobial spectrum test.

The identified bacteria were tested against 54 Gram-positive pathogens from the freezing stocks of LMG, including other strains of *L. garvieae* and important foodborne pathogens listed in the Appendix table 2 and 3. ON cultures of the indicators were first grown at 30 ºC in BHI broth while the bacteria to be tested were incubated in MRS broth at 30 ºC. The following day, 25 µl of the indicator culture was mixed with 5 ml of BHI soft agar before the mixture was poured onto the surface of an agar plate (MRS or BHI). The next step was to spot 3 µl of the bacteria to be tested on top of the indicator lawn. The plates were then incubated aerobically ON at 30 ºC. Among the 54 indicator pathogens, *S. pneumoniae* strain D39 was prepared differently because it produces autolysin, which breaks down its own cells in ON cultures. The bacterium was therefore grown for 4 hours only on the day of the experiment. In preparation for the test, 3 µl from a fresh culture of the bacteria to be tested was spotted on BHI plates and incubated aerobically ON at 30 ºC. On the day of the experiment, 5 ml of TH soft agar inoculated with 25 µl of a fresh culture of *S. pneumoniae* was poured on the medium plates where the isolates had already been spotted the day before. Finally, the plates were incubated anaerobically in a jar with one AnaeroGen 2.5 L Sachet (Thermo Scientific™ Oxoid) at 37 ºC.

2.6 Test of the potential bacteriocins

As mentioned in the introduction, bacteriocins are generally heat stable and protease sensitive peptides. Several tests were therefore conducted to determine whether the antimicrobial substance produced by the isolated bacteria had the mentioned characteristics or not.

2.6.1 **Heat treatment of the supernatant:**

ON (18 h) cultures of all the isolates were first prepared in 5 ml of MRS broth and incubated aerobically at 30 ºC. The bacterial cultures were then centrifuged at 13 000 x g for 10 minutes

before the supernatant was filtrated with a 0.2 µm syringe filter (Sarstedt). The filtrated supernatant was then equally distributed between two Eppendorf tubes. While one tube was incubated at 100 ºC for 5 minutes on a dry block incubator (Thermolyne), the other one was unheated.

2.6.1.1 Microtiter plate assay:

The ability of each isolate to produce the antimicrobial substance in a liquid medium and the effect of heat on the latter was assessed by a microtiter plate assay. The first step was to perform a serial dilution on a 96-well microplate (Sarstedt). Each well was filled with 100 µl of GM17 before 100 µl of the unheated supernatant was added to the first well in the first column of the microtiter plate. The same volume of the heated supernatant was transferred to the second well in the first column. By using a multichannel pipette (Thermo Scientific), a two-fold dilution series of the added samples was made from well 1 to well 11. No sample was added to well 12. In a separate falcon tube, the indicator *L. garvieae* B1678 was diluted 25 times in GM17 before 100 µl was added to all the wells in the microtiter plate. After incubation for 3-4 hours, the absorbance at 600 nm was read by a SPECTROstar ^{Nano} plate reader (BMG LABTECH).

2.6.2 Proteinase K sensitivity

The sensitivity of the antimicrobial substance to proteinase K was tested on an MRS plate on which *L. garvieae* B1678 mixed with 5 ml of MRS soft agar was plated as a lawn. The next step was to spot 3 or 5 µl of an ON culture of the bacteria to be tested at marked places on top of the indicator layer. Then, different volumes (3 µl and 10 µl) of a 20 mg/ml proteinase K was applied near the spotted cultures. If the antimicrobial substance is sensitive to proteinase K, the indicator would be able to grow in the area where the proteinase K was applied.

2.6.3 Production of the antimicrobial substances over time

The production of the antimicrobial substances by the isolates was investigated in MRS and GM17 broths at different time points. Falcon tubes filled with 10 ml of each broth were inoculated with 100 µl of ON cultures before an aerobic incubation at 30 ºC. Samples of 1 ml were then taken after 5 h, 10 h, 15 h, 24 h and 48 h. Each sample was centrifuged, filtrated and immediately stored at - 20 ºC until further use. When all the samples were collected, the antimicrobial activity was determined in a microtiter plate assay as previously described in

2.6.1.1 by using *L. garvieae* B1678 and *L. lactis* B1627 as indicators. The isolates that showed antimicrobial activity in at least 2 wells were then chosen for the protein precipitation step.

2.6.4 Protein precipitation with ammonium sulfate

This step was conducted to check whether the antimicrobials produced by the isolates could be precipitated.

The chosen isolates were incubated at 30 ºC in 50 ml of the liquid medium (1% inoculum) that promoted most antimicrobial production for a specific time determined in 2.6.3. After the incubation, the samples were centrifuged at 10 000 x g for 15 minutes to pellet the cells. The supernatant was transferred to a new falcon tube and a sample of 500 µl was removed for an antimicrobial activity test at a later step. An online ammonium sulfate calculator at http://www.encorbio.com/protocols/AM-SO4.htm was then used to determine the amount of the salt needed to achieve 50 % saturation of the 50 ml supernatant at 4 ºC. After adding 15.05 g of ammonium sulfate to the supernatant, the mixture was incubated ON at 4 ºC. At high concentration of ammonium sulfate, proteins start to aggregate due to the reduced interaction with the water molecules. To harvest the precipitated proteins, the supernatant was centrifuged at 10 000 x g for 30 min (at 4 ºC). The protein pellet was then resuspended in 1 ml of distilled water. Finally, the activity of the unconcentrated supernatant (500 µl) and the concentrated proteins (1 ml) were determined in a microtiter plate as described in 2.6.1.1 by using *L. garvieae* B1678 and *L. lactis* B1627 as indicators.

2.7 Whole genome sequencing

Whole genome sequencing was conducted in order to confirm the existence of bacteriocin genes in the genome of the isolated bacteria. This application requires high quality, high yields and intact DNA fragments to ensure a successful run. Therefore, a milder DNA isolation method than bead beating described in 2.4.1 was chosen.

2.7.1 Extraction of total DNA using enzymes and silica-membrane columns

To extract and purify the total DNA from the pure cultures, the kit DNeasy Blood & Tissue (Qiagen) was used. First, the bacterial cells were harvested and lysed as described in detail in the protocol “pretreatment for Gram-positive bacteria” provided by the manufacturer. The bacterial pellet from 1.5 ml of culture was then resuspended in 180 µl of enzymatic lysis buffer. The latter was made by dissolving lysozyme from chicken egg white (Sigma-aldrich) in Gram-positive

lysis solution (Sigma-aldrich) to a concentration of 45 mg/ml. The suspension was then incubated for 30 minutes at 37 ºC so that the lysozyme could hydrolyze the peptidoglycan in the cell walls. To remove RNA, 20 µl of a RNase A solution (20 mg/ml, Sigma-aldrich) was added before a two-minutes incubation. The next step involved the addition of 20 µl of Proteinase K to digest contaminant proteins. Then, 200 µl of Buffer AL that contained guanidine hydrochloride was added to denature nucleases and to promote the binding of the DNA to the silica column at a later step (Kennedy, 2017). The solution was then vortexed and incubated at 56 ºC for 30 minutes to allow complete lysis. Finally, 200 µl of 96 % ethanol was mixed with the sample to precipitate the DNA.

Another protocol provided by the manufacturer called "Purification of total DNA from Animal Tissues (Spin-Column Protocol)" was followed for the second part of the DNA extraction. The precipitated DNA was transferred to a DNeasy Mini spin column placed in a collection tube, and then centrifuged at 6000 x g for one minute. After discarding the flow though, the spin column was washed with 500 µl of Buffer AW1 (with added 90 % ethanol) and centrifuged as before. A second wash was conducted using Buffer AW2 (with added 90 % ethanol), followed by a centrifugation at 20 000 x g to remove residual ethanol. In the final step, the DNA bound to the silica column was eluted by using 50 µl of Buffer AE (10 mM Tris-Cl and 0.5 mM EDTA pH 9).

To check the result of the extraction, the DNA concentration of each sample was measured by a Qubit ™ fluorometer (Invitrogen). Fluorometric quantifications are more accurate than nanodrop measurements because they measure DNA only. A Qubit ® dsDNA BR Assay Kit (Invitrogen) was used to prepare working solutions (1 µl of dsDNA HS reagent + 199 µl Buffer) and standard solutions (190 µl working solution + 10 µl DNA standard). After reading the standards, a Qubit tube containing 1 µl of the sample and 199 µl of working solution was placed on the fluorometer to be read.

2.7.2 Library preparation and sequencing

Before conducting the whole genome sequencing, the extracted genomic DNA were converted into genomic libraries by ligating adapters that can interact with the sequencing platform.

The library preparation and the sequencing were performed by Cyril Alexander Frantzen according to the protocol for the kit: Nextera ™ Flex Library Preparation (Illumina). This new Illumina product does not require accurate quantification and normalization of genomic DNA

prior to library preparation for samples containing 100 to 500 ng of DNA. In the first step called "tagmentation", the bead-linked transposome (BTL) simultaneously fragments and tags the DNA with Illumina sequencing primers (Head et al., 2014). When the reaction was over, the adapter-tagged DNA were washed to remove any remaining transposomes that might interfere in the next step, which is PCR amplification. As the DNA were amplified, index adapters and sequences required for cluster formations were also added to both ends of the DNA fragments. After purification of the amplified library, no further quantification and normalization was necessary due to the normalization features of the library protocol. Finally, the samples were loaded and sequenced on an Illumina Miseq System.

2.7.3 Analysis of the whole genome sequences

The raw DNA data from the sequencing were stored as FASTQ format and converted to FASTA format files. The latter were annotated on the RAST server (Rapid Annotation using Subsystem Technology). A combination of web-based search tools and publicly available databases were then utilized to analyse the sequences.

To identify putative bacteriocin genes in the genome, the FASTA files were uploaded on BAGEL4, which is the latest updated version of the web-based BActeriocin GEnome mining tooL (BAGEL). The software identifies a set of putative bacteriocin gene clusters based on information from bacteriocin databases and motif databases (de Jong et al., 2006). The putative core peptides obtained in BAGEL4 were then searched in the Protein Basic Local Alignment Search Tool (BLASTp) for similar sequences in the databases. By comparing the results of the search in BLASTp and BAGEL4, the putative bacteriocins were either confirmed or rejected. The online database BACTIBASE was then used for further characterization of the bacteriocins. In cases where BAGEL4 did not detect a putative bacteriocin gene, the annotated genome was searched for the word "bacteriocin" and then BLASTp was used to seek similar sequences. Figure 2.2 shows the steps of the analysis and the programs involved.

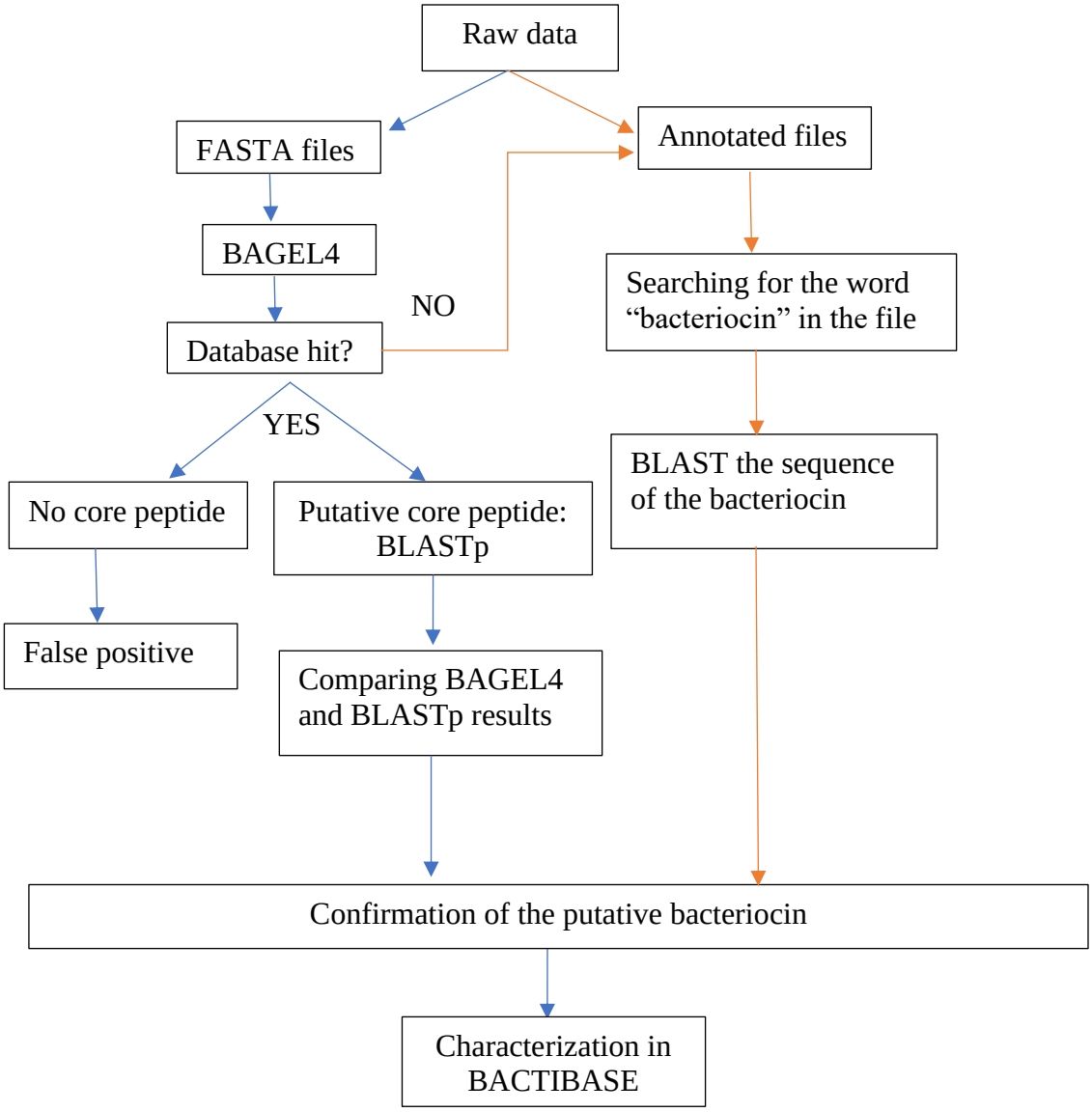


Figure 2.2 The steps in the analysis of the data from the whole genome sequences. The path showed by the blue arrows was first followed. The orange arrows show a second path that was taken in case no results were obtained from the first path (blue arrows).

2.8 Purification and characterization of one potential bacteriocin

Based on the results of all the experiments performed in this study, the potential bacteriocin produced by *Enterococcus thailandicus* was selected to be purified. Although the most frequently used method involves a salt precipitation followed by a combination of ion exchange and reverse phase chromatography (RPC) (Vera Pingitore et al., 2007), the bacteriocin in the study was purified by ammonium sulfate precipitation and one RPC.

To prepare for the precipitation step, 1 liter of MRS broth was inoculated with 10 ml of an ON culture of *E. thailandicus*. The MRS broth was then incubated at 30 ºC for 20 h (determined in 2.6.3) for bacteriocin production. Next, the bacterial culture was centrifuged at 10 000 x g for 10 minutes at 4 ºC to remove the cells. A sample of 1 ml of the supernatant was taken, filtrated and stored at - 20 ºC for a later antimicrobial activity test. The rest of the supernatant was distributed to 3 bottles to facilitate the precipitation step. In the next step, 90.32 g of ammonium sulfate determined by the same online calculator as in 2.6.4, was added to each 330 ml supernatant in the bottles to achieve 50 % saturation. After an ON incubation at 4 ºC, the mixture was centrifuged at 13 800 x g for 50 minutes at 4 ºC to harvest the precipitated proteins. The protein pellets were then dissolved in 100 ml of distilled water, and the supernatant was kept at 4 ºC for a later test. Before further purification, the pH of the protein sample was measured, and it was centrifuged at 10 000 x g for 10 minutes.

A resource RPC column (GE Healthcare Biosciences) connected to an ÄKTA purifier system (Amersham Pharmacia Biotech) was used to further purify the concentrated protein sample. The first step was to wash the 1 ml column with buffer A, which was made by mixing 200 ml of water with 20 µl of trifluoroacetic acid (TFA; Sigma-Aldrich) to maintain the pH. The protein sample was then applied on the column and eluted at increasing concentrations of buffer B. The latter was prepared by mixing 200 µl of isopropanol (Merck) with 20 µl of TFA (Sigma-Aldrich). Finally, the fractions obtained from the RPC, the remaining protein sample, and all the collected supernatants were tested for antimicrobial activity against *L. garvieae* B1678 in a microtiter plate.

To determine the molecular mass of the proteins in the active fractions, an analysis by Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS) was performed by Morten Skaugen. In this technique, the protein samples were first mixed with an UV absorbing matrix to protect the larger molecules before the mixture was ionized by a UV laser in the spectrometer. The generated ions were then separated based on their mass to charge ratio (m/z), which was determined by the spectrometer by measuring the time it took for the ion to travel a certain distance (Singhal et al., 2015).

3. Results

3.1 Screening for bacteriocin producers

Since the purpose of the study was to isolate LAB bacteriocins that could inhibit *L. garvieae* B1678, the latter was used to screen 50 samples of fermented fruits and vegetables in the first round of screening. The screening method consisted of adding three layers of MRS medium to an agar plate: the first one with the diluted samples, the second one was only the medium and the last one was with the indicator. The three dilutions chosen for the samples gave sufficient number of colonies, especially dilution 2 (5 x10^6 times) and dilution 3 (5 x10^8 times). Neat plates with distinct colonies as shown in figure 3.1 were obtained by this method.

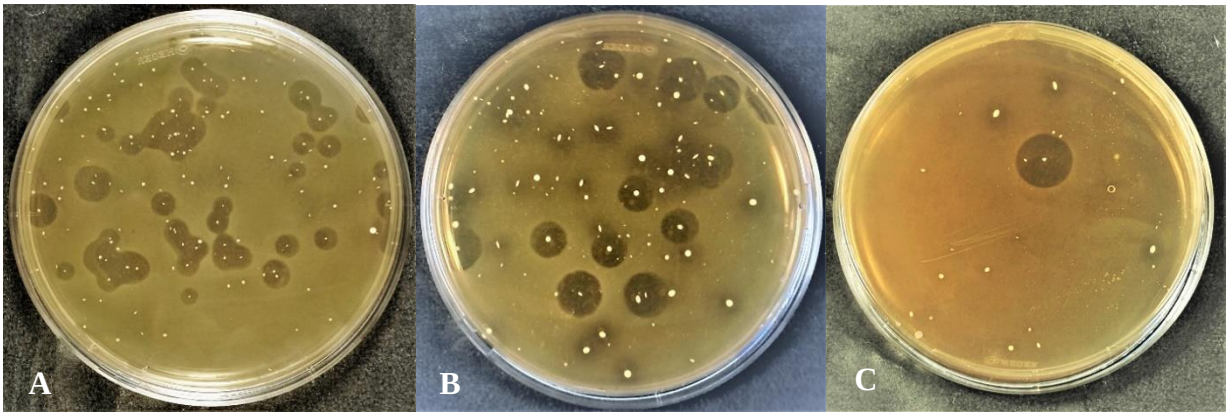


Figure 3.1 Some results from the first screening against *L. garvieae* B1678. (A) Sample 23 +, (B) sample 28 +, and (C) sample 35 +.

Generally, more colonies were observed from the samples that were originally fermented without added NaCl compared to their counterparts with added 0.5 g NaCl (marked by the sign +). Among the 50 samples, 11 of them contained bacteria that inhibited the growth of *L. garvieae* B1678, and 4 out of these 11 samples were originally fermented with added NaCl. The bacteria from the 11 samples were then picked and purified by replating on MRS agar plates. The results from the first screening are summarized in table 3.1.

Table 3.1 The list of the 11 samples containing potential bacteriocin producers. The sign + indicated that the sample was originally fermented with 0.5 g of NaCl

Sample number	Source	Dilution factor
6	Avocado	5×10^8 ***
8	Purple aubergine	5×10^8
15	Ladyfinger/Okra	5×10^4 *
23 +	Karela/Balsam pear	5×10^6 **
28 +	Romanesco broccoli	5×10^4
29	Sugar peas	5×10^8
34	Rambutan	5×10^8
35 +	Eddo/Taro	5×10^6
42	Large chili	5×10^6
48	Dragon fruit (Thanh long)	5×10^8
48 +	Dragon fruit (Thanh long)	5×10^8

*dilution 1
**dilution 2
***dilution 3

The pure cultures were then tested against 2 indicators on different plates in a second round of screening. The same *L. garvieae* B1678 as in the first screening was used again to confirm that the pure cultures can inhibit the bacterium. The nisin Z producer *L. lactis* B1627 was chosen as the second indicator to exclude any nisin producers. Since the latter would be immune to their own bacteriocins, they should not inhibit *L. lactis* B1627.

The results of this second screening showed that all the isolated colonies could inhibit *L. garvieae* B1678 again except the pure cultures from sample 23 +. On the other hand, the isolates from samples 23 + and 15 were the only bacteria that were able to kill *L. lactis* B1627. Figure 3.2 shows the results of the second round of screening for sample 15.

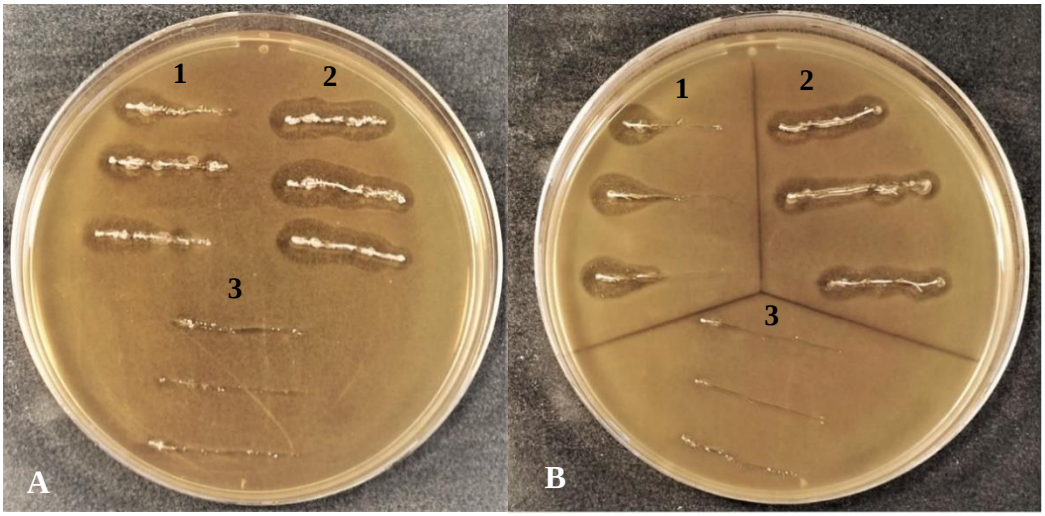


Figure 3.2 Results of the second screening for sample 15, tested against (A) *L. garvieae* B1678; and (B) *L. lactis* B1627. Each column marked by numbers represent one isolate and the different lines are replicates.

The pure cultures that produced clear inhibition zones like in figure 3.2 (A) against *L. garvieae* B1678 on the second screening were selected for further work. In addition, the isolates from sample 23 + were also included because they inhibited *L. lactis* B1627, which indicated that they produced something different from nisin Z. In total, 34 freezing stocks were made.

3.2 Identification of the isolated bacteria

The 34 pure cultures from the screening process were first identified by 16S rRNA gene sequencing and then REP PCR profiling was used to distinguish between the strains.
The 16S rRNA gene from the 34 bacterial samples were sequenced by Sanger sequencing. BLAST was used to search nucleotide databases for similarity. Among the long list of bacteria that matched the DNA sequences, the one with highest identity score and the most repeated name on the list was chosen. The results of the nucleotide search on BLAST showed that 18 out of the 34 bacteria were *L. lactis* strains whereas the remaining 16 bacteria were different species. Since nisin-producing *L. lactis* strains were not the focus of the study, only the identity of the other species is shown in table 3.2. It is observed from the table that the bacteria isolated from the same sample belonged to the same species.

Table 3.2 The identity of 16 isolates based on the 16S rRNA gene sequence

Sample	Given name	Bacteria identity on nucleotide BLAST	Identity %
15	Isolate 1	*Enterococcus faecalis* strain CAU:205	99
	Isolate 2	*Enterococcus faecalis* strain CAU147	97
	Isolate 3	*Enterococcus faecalis* strain ACD47-2	99
	Isolate 4	*Enterococcus faecalis* strain BW1#4	97
23 +	Isolate 5	*Lactococcus garvieae* strain RTCLI04	98
	Isolate 8	*Lactococcus garvieae* strain CAU5908	96
	Isolate 10	*Lactococcus garvieae* strain S1-88	99
28 +	Isolate 1	*Enterococcus faecium* strain CAU7521	98
	Isolate 2	*Enterococcus faecium* strain CAU10445	98
	Isolate 3	*Enterococcus faecium* strain CAU8111	97
	Isolate 4	*Enterococcus faecium* strain CAU7620	98
	Isolate 5	*Enterococcus faecium* strain 17OM39	96
	Isolate 6	*Enterococcus faecium* strain 17OM39	98
	Isolate 7	*Enterococcus faecium* strain CM25	99
34	Isolate 1	*Leuconostoc mesenteroides* strain CAU6332	97
35 +	Isolate 1	*Enterococcus thailandicus* strain CAU3488	97

REP PCR profiling was then conducted to obtain a more accurate distinction between the strains of the identified bacteria. This method generates DNA fingerprints that reflect the distance between repetitive and highly conserved sequences in the bacterial genomes. Since bacterial strains vary in the distance between their consecutive repetitive sequences, a specific strain will be recognizable by its unique profile. Figure 3.3 and 3.4 show the REP PCR profiles obtained from the gel electrophoresis of all the identified bacteria except for *L. lactis* strains.

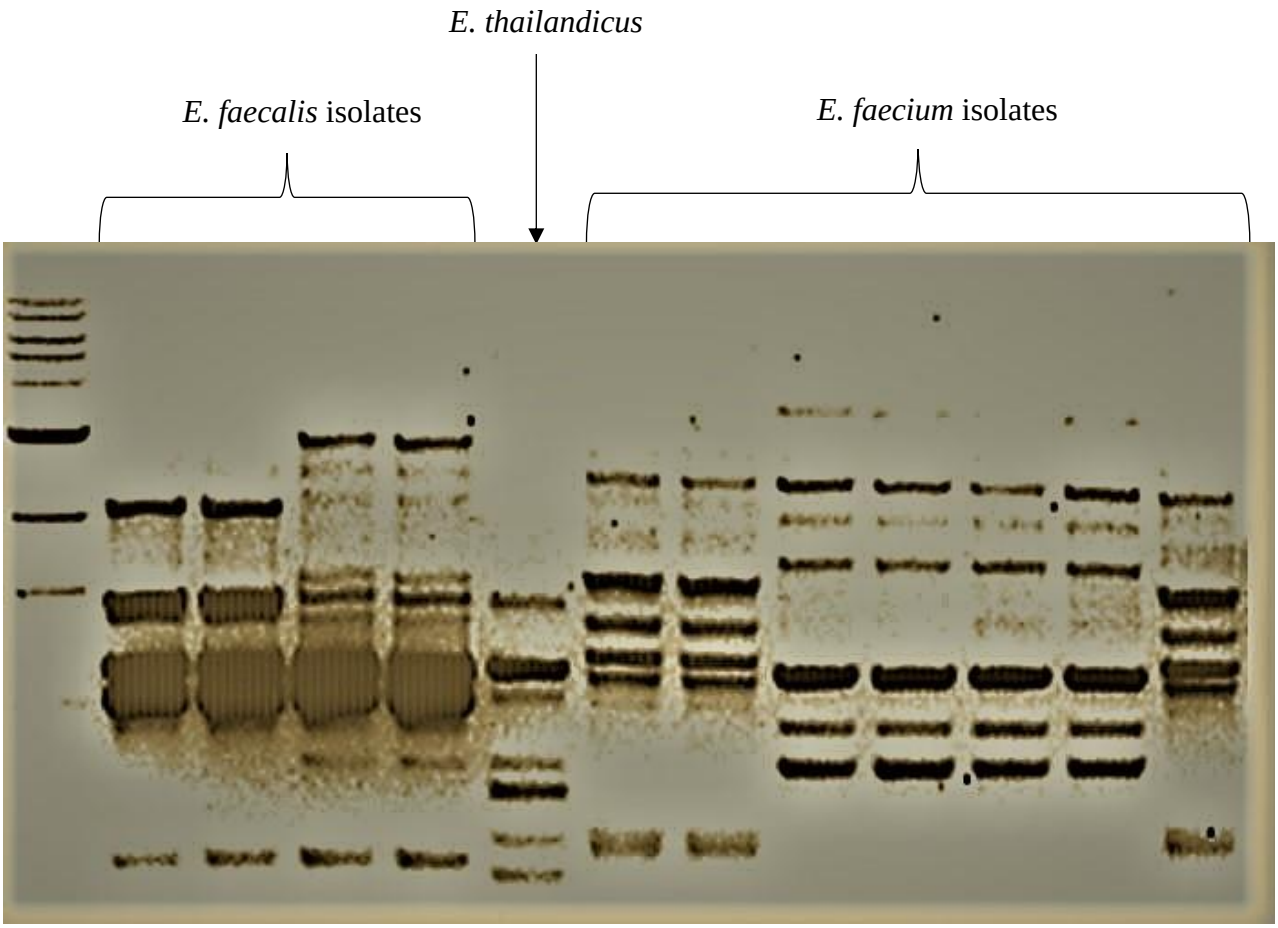


Figure 3.3 The REP PCR profiles of the enterococcal isolates. The first well is 1 kb DNA ladder.

The enterococcal isolates shown in figure 3.3 have their own unique profiles depending on their species. Among the species, identical strains have the same REP PCR profiles. Consequently, the four *E. faecalis* isolates in figure 3.3 can be classified into two separate strains whereas the seven *E. faecium* isolates can be grouped into 2 different strains.

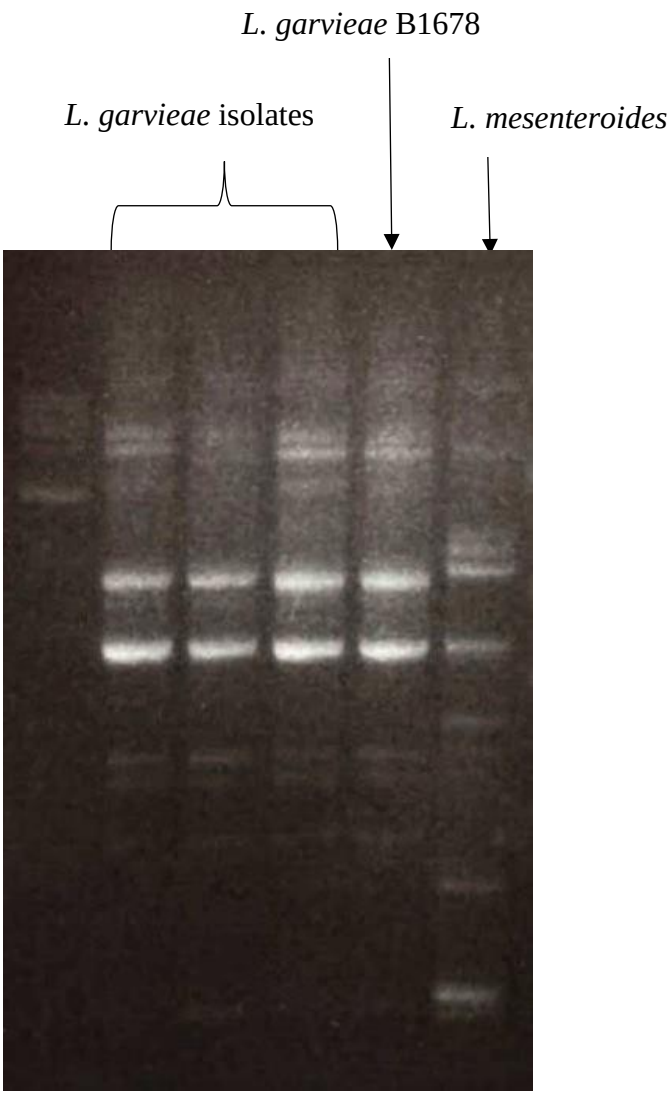


Figure 3.4 REP PCR profiles of the *L. garvieae* isolates, the indicator *L. garvieae* B1678 and *L. mesenteroides.* The first well is 1 kb DNA ladder.

It is observed in figure 3.4 that the *L. garvieae* isolated from the sample 23 + have REP PCR profiles that are identical to that of the indicator *L. garvieae* B1678. This suggests that either the sample 23 + was contaminated with *L. garvieae* B1678 or it did contain *L. garvieae* isolates that are identical to the indicator. To confirm one of the hypothesis, the original stock sample from which the sample 23 + was taken, was screened against *L. garvieae* B1678. The four bacteria that produced clear zones (data not shown) were picked and purified. Then, the pure cultures were tested against *L. garvieae* B1678 and the nisin producer *L. lactis* B1627. Like the isolates from sample 23 +, the purified isolates from the stock sample 23 + did not inhibit *L. garvieae*

B1678 again but killed the nisin producer *L. lactis* B1627. After conducting a 16S rRNA gene sequencing, it was confirmed that the isolates from the stock sample 23 + were *L. garvieae* strains. Due to the unexpected but interesting results, the *L. garvieae* isolated from 23 + were included in the rest of the study. *L. lactis* B1627 was used as their indicator in further tests since the purified isolates do not inhibit *L. garvieae* B1678.

After comparing the REP PCR profiles of the 34 bacteria identified by 16S rRNA gene sequencing, 11 unique profiles corresponding to 11 different strains were selected for further work. These strains are: *E. faecalis* isolate 1 and *E. faecalis* isolate 2 (from sample 15); *L. garvieae* isolate 5 (from sample 23 +); *E. faecium* isolate 1 and *E. faecium* isolate 3 (from sample 28 +); *L. mesenteroides* (from sample 34), *E. thailandicus* (from sample 35 +); 4 strains of *L. lactis* (from sample 6, 29, 42, 48).

3.3 Antimicrobial spectrum of the identified bacteria

Before conducting the inhibition spectrum test, it was important to determine the medium on which the 11 strains produced the most antimicrobials. By using the spot-on-lawn method, *L. garvieae* isolate 5 was tested against *L. lactis* B1627, whereas the other 10 strains were tested against *L. garvieae* B1678 on MRS and BHI plates. The size of the inhibition zones on the two different medium plates was compared. The results (not shown) indicated that the *E. faecalis* strains produced more antimicrobials on BHI plates, whereas MRS plates were more favorable for antimicrobial production by the strains of *E. faecium*, *E. thailandicus* and *L. mesenteroides*. On the other hand, the *L. lactis* strains inhibited the indictor equally on MRS and BHI plates. To obtain optimal results in the inhibition test, the appropriate medium plates was used.

The 11 strains were then tested against 54 Gram-positive pathogens to determine the potential of their antimicrobials in inhibiting other bacteria. Table 3.3 shows the results of the antimicrobial spectrum test for all the strains except for those of *L. lactis* because the latter inhibited the same pathogens as the control (*L. lactis* B1627). The complete test against the 54 indicators is shown in Appendix table 2 and 3. The 21 pathogens included in table 3.3 consist of important foodborne pathogens, other *L. garvieae* strains and *Strep. pneumoniae*.

To assess the level of inhibition against a given indicator, the inhibition zone produced by each strain was given a score from 0 to 3 based on its size compared to the control's (*L. lactis* B1627).

The lowest score 0 corresponded to no inhibition while scores 0.5 to 3 indicated clear and distinct zones of inhibition with varying sizes according to the number.

Table 3.3 The results of the antimicrobial inhibition test against 21 relevant pathogens.

Pathogen names	*E. faecalis*		*L. garvieae* isolate 5	*E. faecium*		*L. mesenteroides*	*E. thailandicus*	*L. lactis* B1627
	isolate 1	isolate 2		isolate 1	isolate 3			
***B. cereus* LMGT2805**	0	0	0	0	0	3	0.5	1
***B. cereus* ATCC 9139 B LMGT2711**	0	0	0	0	0	1	0	1
***B. cereus* 1230, Granum 11-91 LMGT2731**	0	0	0	0	0	2	0.5	1
***B. cereus* ATCC2 (Matforsk) LMGT 2736**	0	0	0	0	0	0	0	1
***L. monocytogenes* LMGT2604**	0	0.5	0	0	0.5	0	1	0
***L. monocytogenes* LMGT2650**	0	0	0	0	0	1	0.5	0
***L. monocytogenes* LMGT2651**	1	1	1	0	0.5	0.5	1	2
L. monocytogenes LMGT2652	1	1	1	0	0.5	1	1	2
***L. monocytogenes* LMGT2653**	1	1	1	0.5	0.5	1	1	2
***S. aureus* LMGT3022**	1	2	0	0	0	0	1	1
***S. aureus* LMGT3023**	0	0	0.5	0	0	1	0	1
***S. aureus* LMGT3242**	0	0	0.5	0.5	0.5	2	0.5	1
***S. aureus* LMGT3262**	0.5	0	0.5	0.5	0.5	1	0.5	1
***S. aureus* LMGT3263**	0	0	0.5	0.5	0.5	1	0.5	1
***S. aureus* LMGT3264**	0	0	0.5	0	0	1	0	1
***S. aureus* LMGT3265**	0	0	0.5	0	0	1	0	1
***Strep. pneumoniae* D39**	2	3	0	0.5	2	0**	2	N/A
***L. garvieae* LMGT3390**	0	0	1	0.5	0.5	0	1	1
***L. garvieae* B1680**	0.5	0.5	1	1	0.5	0.5	1	N/A
***L. garvieae* B1515 (garvicin ML producer)**	1	0.5	1	1	1	0.5	2	N/A
***L. garvieae* B1642 (garvicin KS producer)**	0	0	1	0.5	0	0	1	N/A

** resistant cells covering the original zone
0 = no inhibition; 0.5- 3 = clear and distinct zones

The results of the complete test showed that the control nisin producer (*L. lactis* B1627) and the *L. lactis* strains inhibited 42/54 same indicators, which indicated that the *L. lactis* strains produce nisin. Therefore, only the *L. lactis* isolate 1 from sample 6 was picked for further tests in the rest of the study.

Among the other species, *E. thailandicus* and *L. mesenteroides* were the most effective by killing respectively 33/54 indicators and 32/54 indicators. While *E. thailandicus* strongly inhibited *Strep. pneumoniae* and all the indicator strains of the *L. garvieae*, *L. mesenteroides* was more potent against the foodborne pathogens such as *B. cereus*, *L. monocytogenes* and *S. aureus* as seen in table 3.3. The significant difference between the number of pathogens inhibited by *E. faecium* isolate 1 (19/54 indicators) and *E. faecium* isolate 3 (34/54 indicators) may indicate that they produced different antimicrobials. Although the two strains of *E. faecalis* both inhibited 27/54 indicators, *E. faecalis* isolate 2 produced noticeably bigger zones against the same pathogens. The strains of *E. faecalis* did not always kill the same indicators, which may indicate the production of different antimicrobials. Finally, *L. garvieae* isolate 5 inhibited 28/50 indicators including all the other *L. garvieae* strains listed in table 3.3.

3.4 Test of the potential bacteriocins

3.4.1 Heat stability test

The heat treatment was performed to test whether the antimicrobials produced by the isolated bacteria were heat stable like bacteriocins or not. The supernatants from the bacterial cultures were first heated at 100 ºC for 5 minutes. Then, the antimicrobial activity of the heat-treated supernatant was compared to that of non-heat-treated supernatant in a microtiter plate assay by diluting the samples in a serial two-fold way. No supernatant was added to the last column of the microtiter plate so that it could be used as a control. The wells with OD values smaller than half of that of the control represented clear inhibition against the indicator. The antimicrobial efficiency was therefore represented by the number of wells in which the indicator was inhibited. Table 3.4 summarizes the results of the heat stability test.

Table 3.4 Comparison of the antimicrobial activity in heat treated and non-heat-treated supernatants.

The isolated bacteria	**Sample**	**Indicator**	**Number of well with antimicrobial activity**	
			Non-heated supernatant	Heated supernatant
E. faecalis isolate 1	15	*L. garvieae* B1678	1	1
E. faecalis isolate 2			0	0
E. faecium isolate 1	28 +		2	2
E. faecium isolate 3			1	1
E. thailandicus	35 +		3	3
L. mesenteroides	34		2	2
***L. lactis* isolate 1**	6		5	5
***L. garvieae* Isolate 5**	23 +	*L. lactis* B1627	4	5

Unsurprisingly, *E. faecalis* 1 and *E. faecium* isolate 3 did not produce much antimicrobials in the liquid medium (table 3.4) whereas they showed strong inhibition zones on the medium plates during the screening. The same observation was made for *E. faecalis* isolate 2 that did not produce any antimicrobial substances in the MRS liquid medium (table 3.4). Several studies have reported that LAB bacteria produced more bacteriocins on solid media and less or not detectable bacteriocins in liquid media (Cintas et al., 1995; Maldonado-Barragán et al., 2009). Saucier et al (1995) suggested that the bacterial cells are in closer contact on solid media, which makes it easier for the induction factor that regulates bacteriocin production to reach the cells. The antimicrobial activity observed in one well for *E. faecalis* isolate 1 and *E. faecium* isolate 3 can therefore be caused by lactic acid instead of bacteriocins. For the other strains that inhibited the growth of the indicator in 2 wells or more, the activity can be attributed to the presence of bacteriocins. The antimicrobial substances produced by the latter appeared to be heat stable since the number of wells with inhibition in the heat-treated and non-heat-treated supernatants were the same as seen in table 3.4.

3.4.2 Proteinase K test

To determine the nature of the antimicrobial substances, a proteinase K test on agar plates was conducted. Proteinaceous antimicrobials like bacteriocins are sensitive to proteases.
The indicator was plated as a lawn and the bacteria to be tested were spotted on top of the indicator layer. In the first trial, the same volume (3 µl) of proteinase K (20 mg/ml) and bacteria

were applied to the plates. In the second trial, 10 µl of proteinase K (20 mg/ml) was used for each 5 µl of spotted bacteria. Finally, 3 µl of the most proteinase K resistant bacteria were tested against 10 µl of proteinase K. Some plates on which the proteinase K test was conducted are shown in figure 3.5. The growth of the indicator in the area where proteinase K was applied indicated that the antimicrobials were sensitive to the enzyme.

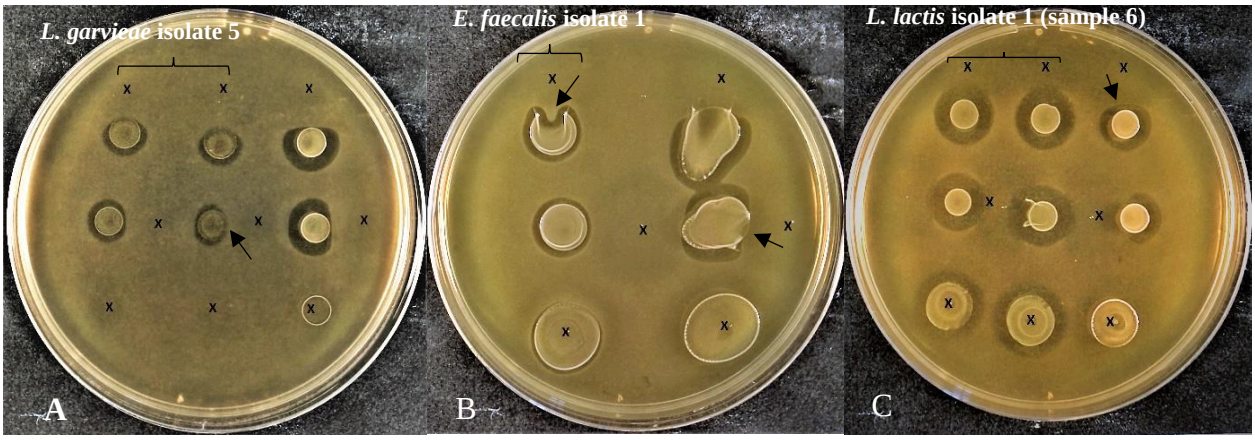


Figure 3.5 The proteinase K test for *L. garvieae* isolate 5 against *L. lactis* B1627 in A; for *E. faecalis* isolate 1 and *L. lactis* isolate 1 against the indicator *L. garvieae* B1678 respectively in B and C. The unmarked last column is the garvicin KS producer *L. garvieae* B1642 used as a control because it is proteinase K sensitive. The proteinase K was applied at the location marked by X, with 3 µl in (A) and 10 µl in (B) and (C). The arrows show a lack of antimicrobial activity, characterized by the growth of the indicator.

The half-moon-shaped zones of inhibition observed in figure 3.5 are typical signs for proteinase K sensitive antimicrobials. Sensitivity to the latter implies that the antimicrobial is proteinaceous. The results of the proteinase K for all the strains summarized in table 3.5.

Table 3.5 Results of the proteinase K test for all the strains

Bacteria	**Proteinase K sensitivity**		
	First trial 3 µl of bacteria + 3 µl proteinase K	Second trial 5 µl of bacteria + 10 µl of proteinase K	Third trial 3 µl bacteria + 10 µl of proteinase K
***E. faecalis* isolate 1**	Not sensitive	Sensitive	N/A
***E. faecalis* isolate 2**	N/A	N/A	N/A
***E. faecium* isolate 1**	Not sensitive	Sensitive	N/A
***E. faecium* isolate 3**	Not sensitive	Sensitive	N/A
Leuconostoc mesenteroides	Not sensitive	Sensitive	N/A
E. thailandicus	Not sensitive	Not sensitive	Not sensitive
***L. garvieae* isolate 5**	Sensitive	N/A	N/A
***L. lactis* isolate 6**	Not sensitive	Not sensitive	Not sensitive

Based on the observation made during the several trials using different volumes of proteinase K and bacterial culture (table 3.5), it was concluded that the volume of proteinase K used should at least be twice as much as that of the spotted bacteria. In fact, in the first trial where equal volume of proteinase K and bacterial cell was applied, only *L. garvieae* isolate 5 was sensitive to the enzyme. On the other hand, four other bacteria including *E. faecalis* isolate 1, *E. faecium* isolate 1 and isolate 3, and *L. mesenteroides* were sensitive to proteinase K in the second trial when 10 µl of the enzyme was used against 5 µl of the bacteria. However, the *L. lactis* isolate 6 and *E.*

thailandicus were always resistant to the enzyme even when 10 µl of proteinase K was added near the 3 µl of spotted bacteria.

3.4.3 Production of the antimicrobial substances over time

To determine the optimum time for antimicrobial production and the liquid medium that promotes most production, 7 different strains were incubated in MRS and GM17 for 48 hours at 30 ºC. A sample of 1 ml was taken after 5 h, 10 h, 15 h, 24 h and 48 h. The antimicrobial activity in the collected samples was determined in a microtiter plate and expressed in BU/ml. One BU is defined as the smallest amount of bacteriocin that can inhibit 50 % or more growth of the indicator strain. Figure 3.6 is a graphical representation of the antimicrobial production in MRS broth over time.

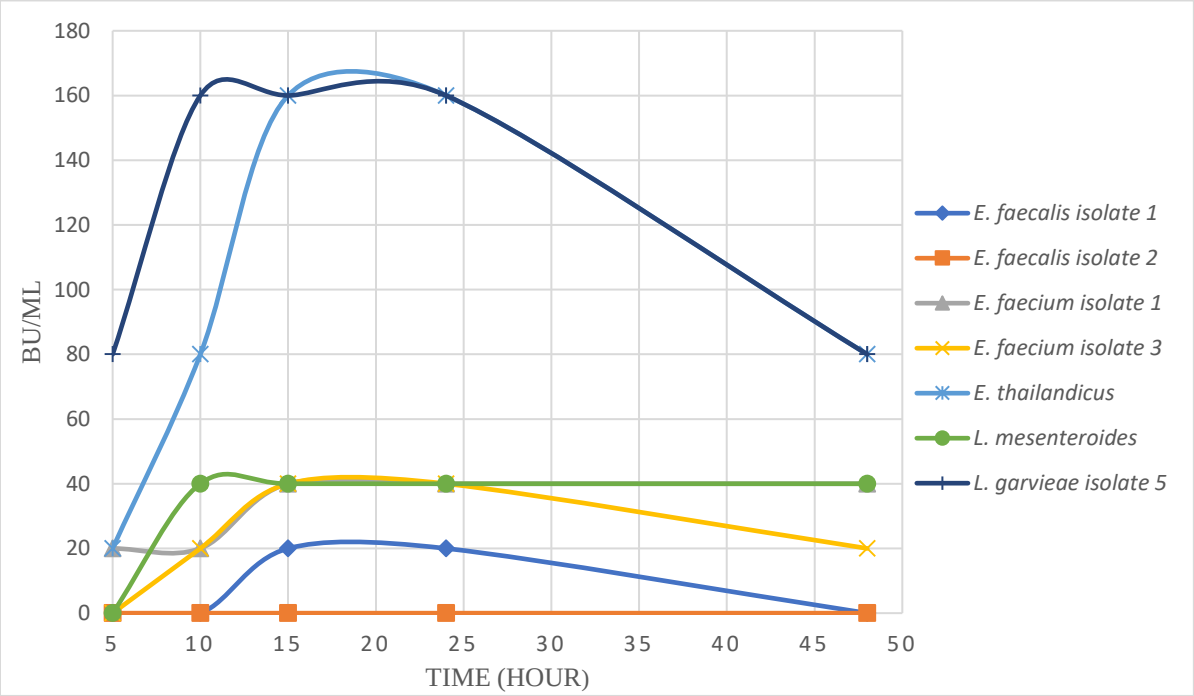


Figure 3.6. Antimicrobial production in MRS broth over time. The indicator used to test *L. garvieae* isolate 5 was *L. lactis* B1627 whereas *L. garvieae* B1678 was used for the other strains.

The general observation in figure 3.6 is that the strains produced the most antimicrobials after an incubation between 15 and 24 hours. However, the production seems to be very low in *E. faecalis* isolate 1 and no antimicrobials was detected from *E. faecalis* isolate 2. There is a slight decrease in the amount of the antimicrobials produced at 48 hours. *E. thailandicus* and *L.*

garvieae isolate 5 had the highest production of antimicrobials during the test in MRS broth. When the 7 strains were incubated in GM17 (data not shown), only *E. thailandicus* had antimicrobial production in of 40 BU/ml between 10 and 15 hours. The other strains did not produce any antimicrobials in GM17. As mentioned before, the production of bacteriocin can be influenced by many factors including medium composition and medium type (solid or liquid).

E. thailandicus and *L. garvieae* isolate 5 were selected for the protein precipitation because they produced most antimicrobials in the present test. *L. mesenteroides* was also chosen because it produced antimicrobials in the present test and had a broad inhibition spectrum in the antimicrobial inhibition test in 3.3.

3.4.4 Protein precipitation

Protein precipitation was performed to further confirm the proteinaceous nature of the antimicrobials produced by *E. thailandicus, L. garvieae* isolate 5 and *L. mesenteroides.* ON cultures of the mentioned bacteria were used to inoculate 50 ml of MRS broth (1% inoculum). The latter were incubated for 20 hours, which was within the incubation time associated with highest antimicrobial production determined in 3.4.3. The supernatant from the cell cultures were then concentrated by adding ammonium sulfate. Finally, the antimicrobial activity of the unconcentrated supernatant and the potential concentrated protein pellet was determined in a microtiter plate. The results of the protein precipitation are shown in table 3.6

Table 3.6 Antimicrobial activity of the precipitated proteins.

The bacterial strains	**BU/ml**		
	Unconcentrated supernatant	Concentrated protein pellet	Supernatant removed from the concentrated pellet
L. mesenteroides	40	320	20
E. thailandicus	160	256	20
***L. garvieae* isolate 5**	160	512	20

The results in table 3.6 indicates that the potential concentrated proteins have much more antimicrobial activity than the unconcentrated supernatant. The antimicrobials produced by these isolates are certainly precipitable. Combined with the results of proteinase K test in 3.4.2, it can

be concluded that the antimicrobials produced by *L. mesenteroides*, *E. thailandicus* and *L. garvieae* isolate 5 are proteinaceous.

3.5 Analysis of the whole genome sequences

The genomes of the 7 potential bacteriocin producers (excluding the nisin producers) were sequenced on a Miseq platform (Illumina) in order to look for the presence of bacteriocin genes. The raw data were then converted to FASTA format and annotated by RAST (Rapid Annotation using Subsystem Technology). To identify the bacteriocin genes in the genome of the 7 sequenced strains, the FASTA files were uploaded on BAGEL4, which is a web based bacteriocin mining tool. The search generally resulted in a table that displayed putative bacteriocin gene clusters called AOI (Area of interest). By clicking on one AOI, a genetic graph of the putative bacteriocin core peptide and all the known genes normally associated with its biosynthesis, such as transporters, regulators, immunity and modification genes, was shown. The putative core peptide was then searched in databases using protein BLAST. Based on the combined information from BAGEL4 and BLASTp, the putative core peptide was either accepted or rejected as a bacteriocin.

The BAGEL4 search was unsuccessful for *E. faecium* isolate 3, therefore the annotated genome was analyzed (semi)-manually. To do so, the word "bacteriocin" was searched in the annotated file, then the corresponding peptide sequence was uploaded on protein BLAST. The best match for the bacteriocin sequence in this genome was the pediocin-like peptide, enterocin TW21 with 98 % identity as shown in table 3.7.

Table 3.7 Output from BLASTp showing the best match for the bacteriocin in the genome of *E. faecium* isolate 3.

enterocin TW21 [Enterococcus faecium]

Sequence ID: AGK85496.1

Score	Expect	Method	Identities	Positives	Gaps
92.4 bits(228)	1e-23	Compositional matrix adjust.	43/44(98%)	44/44(100%)	0/44(0%)

Query	1	MSVSTLGITVDAATYYGNGVYCNTQKCWVDWSRARSEIVDRGVK	44
		+ SVSTLGITVDAATYYGNGVYCNTQKCWVDWSRARSEIVDRGVK	
Sbjct	17	LSVSTLGITVDAATYYGNGVYCNTQKCWVDWSRARSEIVDRGVK	60

The results of the sequence analysis for the other strains are summarized in table 3.8 and their sequence alignments from the search in BLASTp are shown in the Appendix Table 4.

Table 3.8 Analysis of the whole genome sequencing in BAGEL4 and BLASTp.

Organism	**Results of search in BAGEL4**			**BLAST HIT of the core peptide**	**Possible canditate**
	Putative bacteriocins	Core peptide	Genes involved in the bacteriocin biosynthesis		
***E. faecalis* Isolate 1**	Microcin N from *Escherichia coli*	Yes	HlyD: transporter	47 % microcin from *E. coli*	Yes
				100 % microcin 24 from *Yersinia ruckeri* ATCC 29473	
***E. faecalis* Isolate 2**	Enterocin W alfa	Same core peptide	ABC transporter LanM: lantibiotic modifying enzyme LanT: lanthibiotic transport system	98 %	Yes
	Plantaricin W			51%	No
	Enterocin W beta	Yes		100 %	Yes
	Enterolysin A	Yes	None	100 %	Yes
***E. faecium* Isolate 1**	Enterolysin A	Yes	None	99 % peptidase M23 from *E. faecium*	Yes
	Carnocyclin A	Yes	ABC transporter	95 % enterocin NKR-5-3B from *E. faecium*	Yes
E. thailandicus	Enterocin NKR-5-3B	Yes	ABC transporter	100 % thaiocin 1: circularin A/ uberolysin family	Yes
L. mesenteroides	Enterocin L50b from *E. faecium* L50	Yes	None	65 % enterocin L50 from *E. faecium*	Yes
***L. garvieae* Isolate 5**	Garvieacin Q	Yes	HlyD: transporter LanT: leader cleavage	100 %	Yes

A few of the putative bacteriocins identified by BAGEL4 did not have any core peptide and were therefore excluded from table 3.8. The genomic analysis of the strains listed in the table from BAGEL4 indicated that all of them had candidate bacteriocin genes. The search of the core peptide in protein BLAST gave additional information that were used to confirm or reject the putative bacteriocin gene found in BAGEL4. The following paragraphs justifies the confirmation of the putative bacteriocin in each genome shown in table 3.8.

***E. faecalis* isolate 1:** The sequence for microcin N identified by BAGEL4 in this genome was found to be 100% identical to microcin 24 (old name for microcin N) from *Y. ruckeri* ATCC 29473 in BLASTp. Therefore, the latter was confirmed as a possible candidate.

***E. faecalis* isolate 2**: BAGEL4 identified 3 AOIs in this genome. In the first one, different bacteriocins (Enterocin W alfa and Plantaricin W) were assigned to the same core peptide. However, the search of the core peptide in BLASTp revealed that it was more similar to enterocin W alfa (98 % identity) than plantaricin W (51 % identity). The second AOI contained the core peptide for enterocin W beta, which had 100 % sequence identity to a corresponding bacteriocin in BLASTp. Since enterocin W alfa and enterocin W beta are the components of the two-peptide bacteriocin enterocin W (Sawa et al., 2012), it makes sense more to confirm enterocin W alfa instead of plantaricin W.
The last AOI consisted only of the core peptide for enterolysin A without other associated genes shown. The bacteriocin is a possible candidate because the search of the core peptide in BLASTp had a 100 % identity match with enterolysin A (class III bacteriocin) produced by *E. faecalis*.

***E. faecium* isolate 1:** The results from BAGEL4 showed 2 AOI in this genome. In the first one, only a core peptide for enterolysin A without genes involved in bacteriocin biosynthesis was detected. The search on BLASTp showed that the putative bacteriocin peptide shared 99 % identity with an M23 peptidase from *E. faecium*. The M23 peptidases are a family of enzyme that lyse the peptidoglycans in the bacterial cell wall. Considering that enterolysin A belong to the M23/M37 family of zinc metallopeptidase (Khan et al., 2013), it could be acceptable to assume that *E. faecium* isolate 1 contain enterolysin A. In addition, Suárez et al (2015) detected the enterolysin A gene in the genome of several *E. faecium* strains despite the bacteriocin being only characterized in *E. faecalis* previously.

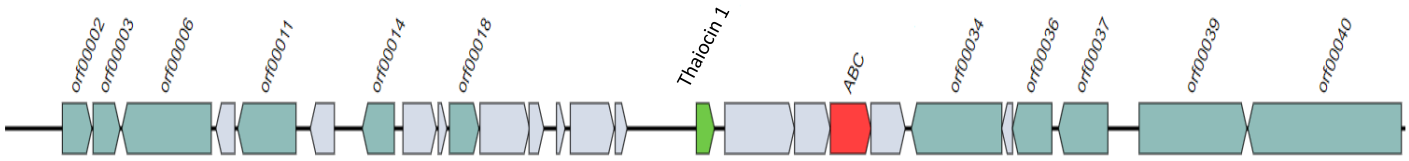
The second AOI in the genome of *E. faecium* contained a putative core peptide for carnocyclin A in BAGEL4. However, the results in BLASTp showed 95 % identity to the circular enterocin NKR-5-3B produced by *E. faecium.*

E. thailandicus: The genome of this bacterium contained a gene that encoded for enterocin NKR-5-3B and a gene for an ABC transporter according to the results from BAGEL4. The search of the core peptide in BLASTp indicated that the bacteriocin gene showed 100 % identity to thaiocin 1, a circular bacteriocin produced by *E. thailandicus.*

L. mesenteroides: BAGEL4 only identified the gene for the core peptide of enterocin L50b in this genome. The results of the search in BLASTp revealed that the bacteriocin sequence shared 65 % sequence identity to that of enterocin L50 from *E. faecium.*

***L. garvieae* isolate 5:** This genome contained a gene for garvieacin Q and 2 genes associated with bacteriocin biosynthesis as shown in table 11. The putative bacteriocin was confirmed because the results in BASTp indicated that it shared 100 % sequence identity to garvieacin Q found in the database.

The next step in the study was to select 1 of the 7 sequenced strains for further characterization and for the last purification step. *E. thailandicus* was chosen because it had the broadest spectrum of activity in the inhibition test and was very effective against all the *L. garvieae* strains. Moreover, the circular bacteriocin (thaiocin 1) from *E. thailandicus* has not been thoroughly studied or characterized in research papers.
The putative genes responsible for the production of thaiocin 1 identified by BAGEL4 are shown in figure 3.7.



GENE NAME	FUNCTION
ORF00002	50S ribosomal protein L13
ORF00003	30S ribosomal protein S9
ORF00006	Tyrosine recombinase XerC
ORF000011	Phasyl DNA replicon protein arp
ORF000014	HTH-type transcriptional regulator Xre
ORF000018	Putative bacteriocin transporter
THAIOCIN 1	Core peptide
ABC	Nod factor export ATP-binding proteins I
ORF000034	Bacteriocin production related histidine kinase
ORF000036	Protein FsrB
ORF000037	Response regulator protein
ORF000039	Capsule synthesis positive regulator AcpB
ORF000040	Probable cation-transporting ATPase F

Figure 3.7 Representation of the thaiocin gene cluster from BAGEL4. The gene names are shown in the figure whereas their functions are listed below it. Only the putative genes with known functions are shown.

It is observed from figure 3.7 that the essential genes for bacteriocin production are present in the cluster, namely the structural gene for thaiocin 1 and the ABC immunity/transport.

The theoretical molecular mass of thaiocin 1 can be predicted by analyzing its amino acid sequence and by comparing it to that of a known circular bacteriocin. Amylocyclin produced by *Bacillus amyloliquefaciens* FZB42 has been described as having similarity to the circular nonlantibiotic bacteriocins produced by lactic acid bacteria (Scholz et al., 2014). Therefore, amylocyclin was used as a model for the determination of the theoretical mass of thaiocin 1 in table 3.9. Some important information about the peptide sequences of amylocyclin and thaiocin 1 are also shown in the table. The molecular mass of the mature linear peptide was determined by uploading the protein sequence in BACTIBASE. On the other hand, the molecular mass of the

mature circular amylocyclin was retrieved from the study by Scholz et al. (2014). It was observed that one water molecule was removed from the linear peptide during the circularization of amylocyclin. Therefore, the theoretical molecular weight of thaiocin was determined by subtracting the mass of one water molecule from its mature linear peptide.

Table 3.9 Steps in the determination of the theoretical mass of thaiocin 1.

Amylocyclin	
Leader peptide MNLVKSNKKSFILFGAALAAATLVYALLLTGTELNVAAAHAFSANAELASTLGISTAAAKKAI DIIDAASTIASIISLIGIVTGAGAISYAIVATAKTMIKKYGKKYAAAW	
Mature linear peptide	LASTLGISTAAAKKAIDIIDAASTIASIISLIGIVTGAGAISYAIVATAKTMIK KYGKKYAAAW Molecular mass: 6400 Da
Mature circular peptide	LASTLGISTAAAKKAIDIIDAASTIASIISLIGIVTGAGAISYAIVATAKTMIK KYGKKYAAAW Molecular mass: 6381 Da
Change in mass during circularization	6400 Da – 6381 Da = 19 Da 19 Da or 18 Da = 1 water molecule
Thaiocin	
Leader peptide MKKNLLLVLPIVGIVGLFVGAPMLTANLGISSYAAKKVIDIINTGSAVATIIALVTAVVGGGLIT AGIVATAKSLIKKYGAKYAAAW	
Mature linear peptide	LTANLGISSYAAKKVIDIINTGSAVATIIALVTAVVGGGLITAGIVATAKSLI KKYGAKYAAAW Molecular mass: 6335 Da
Mature circular peptide	LTANLGISSYAAKKVIDIINTGSAVATIIALVTAVVGGGLITAGIVATAKSLI KKYGAKYAAAW **Theoretical molecular mass:** 6335 Da – 19 Da = **6316 Da**

3.6 Purification and characterization of the bacteriocin from *E. thailandicus*

In the last step of the study, the bacteriocin produced by *E. thailandicus* was purified and characterized. A combination of ammonium sulfate precipitation and reverse-phase chromatography (RPC) was performed for this purpose. The antimicrobial activity of the

collected supernatants, the concentrated proteins and the 31 fractions obtained from the RPC was then determined against *L. garvieae* B1678 in a microtiter plate. The results of the test are shown in table 3.10

Table 3.10 Determination of the antimicrobial activity in the samples from the steps of the purification

Sample	**Initial volume in ml**	**Number of wells with activity**	**BU in 50 µl**	**BU/ml**	**Total activity (BU)**	**Recovery (%)**
Culture supernatant	1000	3	4	80	80 000	100
Supernatant removed from the protein precipitates	1000	2	2	40	40 000	50
Concentrated proteins (pH 4.5)	100	6	32	640	64 000	80
Fractions from RPC						
Fractions	**Initial volume in ml**	**Number of wells with activity**	**BU in 10 µl**	**BU/ml**	**Total activity (BU)**	**Recovery** (%)
F1	1	4	8	800	800	1
F2	1	9	256	25600	25600	32
F3	1	7	64	6400	6400	8
F4 to F8	1	4	8	800	800	1
F9 to 31	1	1	1	100	100	0.1

It is observed from table 3.10 that the proteins are more active as they become purer. In fact, F2 from the RPC has an activity of 25600 BU/ml compared to the concentrated proteins with 640 BU/ml. However, there is a loss of total activity in further steps of the purification as illustrated by 32 % recovery in F2 compared to 80 % recovery at the precipitation step. The activity observed in the supernatant removed from the protein precipitates clearly indicated that some proteins were lost during the process.

Among the 31 fractions obtained from the RPC, F1 to F4 showed antimicrobial activity (table 3.10). These fractions were eluted with isopropanol concentration between 17 % and 25 % (data not shown). Fraction 2 that exhibited the highest antimicrobial activity was eluted at 20 % isopropanol. The rest of the fractions that had antimicrobial activity in only one well were discarded while the four active fractions were further characterized. The molecular mass of the purified proteins in the 4 active fractions (F1 to F4) was then determined by MALDI TOF MS. The results are displayed in Figure 3.8 for F1, F2 and F3 because a peptide close to the theoretical size (6316 Da) determined in table 3.9 was detected in these fractions.

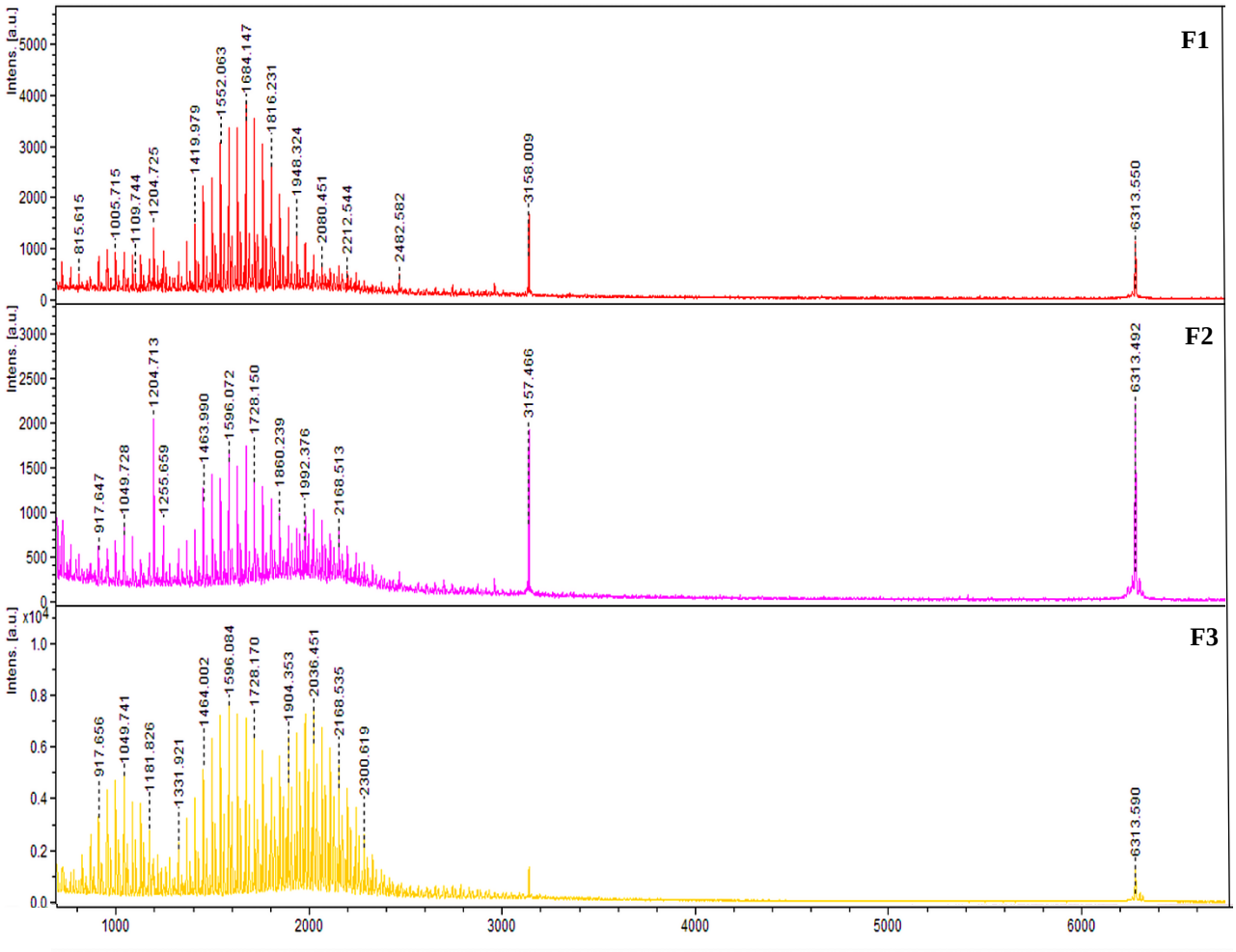


Figure 3.8 MALDI TOF MS analysis of F1, F2 and F3 after one RPC. The m/z ratio shown in the x axis represents the molecular mass in Da whereas the intensity signal in the y axis corresponds to the amount of the peptide.

Several peaks are observed in figure 3.8 but the last one in all the fractions, which is the largest peptide with a molecular mass around 6313 Da is most likely to represent the circular bacteriocin thaiocin 1. The single peak at 3157.466 in F2 is a doubly charged $[M + 2H]^{2+}$ variant of the singly charged peptide $[M+ H]^{+1}$ at 6313.492. The same observation is made in F1 and F3. Based on the MALDI TOF mass spectrum, the practical molecular mass of thaiocin 1 is 6312 Da after subtracting 1 Da equivalent to the mass of one proton.

4. Discussion

The purpose of the study was to isolate and characterize bacteriocins produced by lactic acid bacteria that could be used against the fish pathogen *L. garvieae.* Among the 50 samples of fermented vegetables and fruits screened, bacteriocin producers were found in 11 samples. With the exception of romanesco broccoli (sample 28) that had Italian origin, the other 10 samples containing bacteriocin producers were from tropical fruits and vegetables. Although more colonies were observed from the samples fermented without NaCl, the presence of bacteriocin producers in samples 23 +, 28 + and 35 + and not their counterpart (table 3.1) may indicate that their bacteriocin production is influenced by salt in their environment. Further investigations about the effect of salt on the isolates from these samples must be performed before drawing any conclusions.

After the first screening, the pure cultures isolated were tested against the same indicator *L. garvieae* B1678 to confirm their inhibitory activity and against *L. lactis* B1627 to exclude nisin producers. The test against *L. garvieae* B1678 revealed that all the pure cultures except for the ones from sample 23 + inhibited the indicator. According to the test against *L. lactis* B1627, all the pure cultures except the ones from sample 23 + and 15 were nisin producer due to their lack of inhibition against the indicator. While some of these strains were identified by 16S rRNA gene sequencing (table 3.2) as non-nisin producers in the species of *E. faecium* (28 +), *E. thailandicus* (35 +) and *L. mesenteroides* (34), the rest were indeed *L. lactis* strains. The only bacteria mentioned earlier that inhibited *L. lactis* B1627 were strains of *E. faecalis* (15) and *L. garvieae* (23 +) as shown in table 3.2. In conclusion, a lack of inhibition against *L. lactis* on plates does not necessarily confirm that the bacteria produce nisin. A 16S rRNA gene sequencing should always be performed to determine the identity of the bacteria.

The REP PCR profiling provided a clear and reliable distinction between the different strains of the identified bacteria as shown in figure 3.3 and 3.4. Interestingly, the *L. garvieae* strains (from sample 23 +) had an identical REP PCR profile to that of the indicator strain *L. garvieae* B1678. Since the screening of the original 23 + stock sample resulted in the identification of *L. garvieae* isolates, it is unlikely that sample 23 + was contaminated by *L. garvieae* B1678. The conclusion is that sample 23 + contained *L. garvieae* that were identical to the indicator used. These *L. garvieae* isolated from 23 + can inhibit the indicator in mixed cultures, but they are unable to kill it again when purified. It can be speculated that large amount of bacteriocins are produced in

colonies/mixed cultures, which overrides immunity in the indicator strain *L. garvieae* B1678 thereby causing its death.

Based on their unique REP PCR profiles, the following 7 strains were selected for further characterization: *E. faecalis* isolate 1, *E. faecalis* isolate 2, *E. faecium* isolate 1, *E. faecium* isolate 3, *E. thailandicus*, *Leuc. mesenteroides* and *L. garvieae* isolate 5. The antimicrobial spectrum of these isolates was assessed by comparing their inhibition zones against a given indicator to that of the control and by giving scores from 0 to 3 accordingly. Although the method is simple and fast, it relies heavily on the personal observation of the student. To obtain objective results, the diameter of the inhibition zone should be measured.

The genomes of these 7 strains were sequenced, then analyzed by using BAGEL4 and BLASTp as shown in table 3.8. The results showed that each genome contained putative bacteriocin genes. The different bacteriocins identified illustrates well the diversity of lactic acid bacteriocins. In the case of *E. faecalis* isolate 2 and *E. faecium* isolate 1, two putative bacteriocins belonging to different classes were identified in both genome. The significantly bigger zones produced by *E. faecalis* isolate 2 compared to those produced by *E. faecalis* isolate 1 during the inhibition test might be attributed to the action of two bacteriocins. However, it is not known if the two bacteriocins were produced simultaneously during the experiment. The significant difference in the inhibition spectrum of *E. faecium* isolate 1 and *E. faecium* isolate 3 could be explained by the different bacteriocins they produce. Nevertheless, further studies about the expression of the putative bacteriocins in the genome of these strains must be conducted before drawing conclusions.

Due to limited time of the thesis, the bacteriocins found to be well characterized in literatures are omitted whereas the less characterized and potentially new ones are highlighted in this section.

The genome of *E. faecalis* isolate 1 contained a peptide sequence that showed 47 % sequence identity to microcin N previously known as microcin 24 produced by *E. coli* and 100 % identity to the same bacteriocin from *Y. ruckeri* ATCC 29473 (table 3.8). Microcin is a family of bacteriocins principally reported in Gram-negative Enterobacteria that are grouped into class I and class II (Kaur et al., 2016). Microcin N found in the genome of *E. faecalis* isolate 1 belongs to class II that consists of unmodified or subtly modified peptides. Although the latter are not well characterized in literatures, a study by Wooley et al. (1999) provided valuable information

about the antimicrobial spectrum of microcin N produced by *E. coli* AvGOB18. The authors reported that microcin N inhibited the growth of 7 serotypes of Salmonella and 7 strains of *E. coli* O157:H7 but was ineffective against several strains of *L. monocytogenes* and *Campylobacter jejuni* (Wooley et al., 1999). In contrast, the inhibition spectrum test of the present study (table 3.3) showed that *E. faecalis* isolate 1 inhibited 3/5 strains of *L. monocytogenes.* In addition, the antimicrobial was strongly effective against *S. pneumoniae* D39, which is relevant for studies on antibiotic-resistant *S. pneumoniae*. Microcin N has been reported to be sensitive to proteinase K (Corsini et al., 2010), which was supported by the results of the proteinase K test in figure 3.5 (B). Regardless of the compelling results indicating the production of microcin N by *E. faecalis* isolate 1, gene knockout experiments and a purification step should be conducted to confirm the suppositions. The inhibition spectrum could also be further investigated by performing tests against Enterobacteria.

In the genome of *L. mesenteroides,* a peptide sequence sharing 65 % identity with enterocin L50 from *E. faecium* was found. Reported bacteriocins produced by *Leuconostoc* include leucocins and mesenterocins (Wan, 2017). To the best of found knowledge, no enterocin L50 has been reported in *L. mesenteroides.* Enterocin L50 produced by *E. faecium* L50 consists of two leaderless peptides, enterocin L50A and L50B with 72 % sequence identity (Cintas et al., 1998) . Interestingly, BAGEL4 only identified one core peptide belonging to enterocin L50B. The combined activity of enterocin L50 has been reported to inhibit strains of *Enterococcus, Lactobacillus, Lactococcus lactis, Pediococcus pentosaceus* and the foodborne pathogens *L. monocytogenes* and *B. cereus* (Franz et al., 2007). In comparison, *L. mesenteroides* in the present study strongly inhibited 3/4 strains of *B. cereus*, 4/5 strains of *L. monocytogenes* and 6/7 strains *of S. aureus.* The cell free supernatant from *L. mesenteroides* was heat stable and sensitive to proteinase K. To further characterize the potential enterocin L50 produced by this bacterium, a purification step followed by N-terminal sequencing could be performed.

The bacteriocin found in the genome of *E. thailandicus* is a circular one in the family circularin A/ uberolysin (Table 3.8) that was named thaiocin 1 because *E. thailandicus* was first isolated from fermented sausages (“mum”) in Thailand (Tanasupawat et al., 2008). The conformation of circular bacteriocins makes them resistant to several proteases and to wide range of temperatures according to Belkum et al. (2011). The proteinase K and heat treatment tests conducted in this study supported the assumption that thaiocin was heat stable and proteinase K resistant (table

3.5). Although the studies about thaiocin 1 are rare, Lin et al. (2013) reported its broad activity spectrum by the inhibition of 22 indicators including strains of *L. garvieae*, *S. aureus* and *L. monocytogenes*. The results of the inhibition test (table 3.3) in this study are consistent with these findings with the observation that *E. thailandicus* inhibited all the 5 strains of *L. monocytogenes* and 4 /7 strains of *S. aureus*. In addition to having the broadest inhibition spectrum in the test, the bacterium inhibited all the 4 strains of *L. garvieae.* Considering the aim of the study and the results of all the tests conducted, the bacteriocin from *E. thailandicus* was selected for the last purification step. The MALDI TOF analysis of the purified bacteriocin revealed that thaiocin 1 had a molecular mass of 6312 Da, which was close to the theoretical mass determined at 6316 Da (table 3.9). However, Lin et al. (2013) reported a molecular mass of 6319 Da. The difference of 7 Da could be attributed to different purification procedure, different MS instruments used or inaccurate reading of the mass spectrum data.

With respect to the aim of the study, thaiocin 1 from *E. thailandicus* is the best candidate to fight *L. garvieae* infections in fish since it strongly inhibited all the *L. garvieae* strains tested. In addition, its effectiveness against *L. monocytogenes* can be exploited in the food industry. To evaluate the potential of thaiocin 1 in medicines, its inhibition spectrum against clinical pathogens need to be determined. The other bacteriocins identified in the study also showed inhibition against the tested *L. garvieae strains*, but to a lesser degree than thaocin 1. On the other hand, many of them were effective against foodborne pathogens, which is an advantage in the food industry and in the treatment of foodborne diseases. Studies focusing on these other bacteriocins should be performed to further characterize them.

Diverse methods were used throughout the study from the screening process to the purification step. The molecular techniques including 16S rRNA gene analysis, REP PCR profiling, the whole genome sequencing and MALDI TOF MS were essential to answer many questions along the study. They gave reliable results and provided an insight at the genetic level. However, the information from these techniques cannot be verified without practical tests. In fact, the antimicrobials identified in the genome could not be well characterized without the spot-on-lawn inhibition assay, the proteinase K and heat stability tests. Therefore, molecular techniques with conventional methods constitute the best way to study bacteriocins.

References

Allen, H. K., JulianTrachsel, Looft, T. & Casey, T. A. (2014). Finding alternatives to antibiotics. *Annals of the New York Academy of Sciences*, 1323 (1): 91-100. doi: doi:10.1111/nyas.12468.

Alvarez-Sieiro, P., Montalbán-López, M., Mu, D. & Kuipers, O. P. (2016). Bacteriocins of lactic acid bacteria: extending the family. *Applied Microbiology and Biotechnology*, 100 (7): 2939-2951. doi: 10.1007/s00253-016-7343-9.

Arabestani, M. R., Mousavi, S. M. & Alikhani, M. Y. (2014). Bacteriocins as the Alternatives to Antibiotics. *Avicenna J Clin Microb Infec*, 1 (2): e21086. doi: 10.17795/ajcmi-21086.

Barnett, T. C., Cole, J. N., Rivera-Hernandez, T., Henningham, A., Paton, J. C., Nizet, V. & Walker, M. J. (2015). Streptococcal toxins: role in pathogenesis and disease. *Cell Microbiol*, 17 (12): 1721-41. doi: 10.1111/cmi.12531.

Bemena, L. D., Mohamed, L. A., Fernandes, A. M. & Lee, B. H. (2014). Applications of bacteriocins in food, livestock health and medicine. *International Journal of Current Microbiology and Applied Sciences* 3(12): 924-949.

Bernheimer, A. W. (1966). DISRUPTION OF WALL-LESS BACTERIA BY STREPTOCOCCAL AND STAPHYLOCOCCAL TOXINS. *Journal of Bacteriology*, 91 (5): 1677-1680.

Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research*, 7 (6): 1514.

Brumfitt, W., Salton, M. R. J. & Hamilton-Miller, J. M. T. (2002). Nisin, alone and combined with peptidoglycan-modulating antibiotics: activity against methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococci. *Journal of Antimicrobial Chemotherapy*, 50 (5): 731-734. doi: 10.1093/jac/dkf190.

Chambers, H. F. & DeLeo, F. R. (2009). Waves of Resistance: Staphylococcus aureus in the Antibiotic Era. *Nature reviews. Microbiology*, 7 (9): 629-641. doi: 10.1038/nrmicro2200.

Cintas, L. M., Rodriguez, J. M., Fernandez, M. F., Sletten, K., Nes, I. F., Hernandez, P. E. & Holo, H. (1995). Isolation and characterization of pediocin L50, a new bacteriocin from Pediococcus acidilactici with a broad inhibitory spectrum. *Applied and Environmental Microbiology*, 61 (7): 2643-2648.

Cintas, L. M., Casaus, P., Holo, H., Hernandez, P. E., Nes, I. F. & Håvarstein, L. S. (1998). Enterocins L50A and L50B, Two Novel Bacteriocins from Enterococcus faecium L50, Are Related to Staphylococcal Hemolysins. *Journal of Bacteriology*, 180 (8): 1988-1994.

Clarridge, J. E., 3rd. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin Microbiol Rev*, 17 (4): 840-62, table of contents. doi: 10.1128/CMR.17.4.840-862.2004.

Coates, A. R. M., Halls, G. & Hu, Y. (2011). Novel classes of antibiotics or more of the same? *British Journal of Pharmacology*, 163 (1): 184-194. doi: 10.1111/j.1476-5381.2011.01250.x.

Collins, M. D., Farrow, J. A., Phillips, B. A. & Kandler, O. (1983). Streptococcus garvieae sp. nov. and Streptococcus plantarum sp. nov. *J Gen Microbiol*, 129 (11): 3427-31. doi: 10.1099/00221287-129-11-3427.

Corsini, G., Karahanian, E., Tello, M., Fernández, K., Rivero, D., Saavedra, J. & Ferrer, A. (2010). *Purification and characterization of the antimicrobial peptide microcin N*, vol. 312.

Cotter, P. D., Ross, R. P. & Hill, C. (2013). Bacteriocins - a viable alternative to antibiotics? *Nat Rev Microbiol*, 11 (2): 95-105. doi: 10.1038/nrmicro2937.

de Jong, A., van Hijum, S. A., Bijlsma, J. J., Kok, J. & Kuipers, O. P. (2006). BAGEL: a web-based bacteriocin genome mining tool. *Nucleic Acids Res*, 34 (Web Server issue): W273-9. doi: 10.1093/nar/gkl237.

Dimov, S., Ivanova, P. & Harizanova, N. (2005). Genetics of Bacteriocins Biosynthesis by Lactic Acid Bacteria. *Biotechnology & Biotechnological Equipment*, 19 (sup2): 4-10. doi: 10.1080/13102818.2005.10817270.

Dr.Biology. (2010). *Alkaline Lysis*: Arizona State University School of Life Sciences Ask A Biologist. Available at: https://askabiologist.asu.edu/alkaline-lysis (accessed: April 13, 2018).

Eijsink, V. G., Axelsson, L., Diep, D. B., Havarstein, L. S., Holo, H. & Nes, I. F. (2002). Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie Van Leeuwenhoek*, 81 (1-4): 639-54.

Eraclio, G., Ricci, G., Quattrini, M., Moroni, P. & Fortina, M. G. (2018). Detection of virulence-related genes in Lactococcus garvieae and their expression in response to different conditions. *Folia Microbiol (Praha)*, 63 (3): 291-298. doi: 10.1007/s12223-017-0566-z.

Franz, C. M., van Belkum, M. J., Holzapfel, W. H., Abriouel, H. & Galvez, A. (2007). Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. *FEMS Microbiol Rev*, 31 (3): 293-310. doi: 10.1111/j.1574-6976.2007.00064.x.

Fujita, K., Ichimasa, S., Zendo, T., Koga, S., Yoneyama, F., Nakayama, J. & Sonomoto, K. (2007). Structural analysis and characterization of lacticin Q, a novel bacteriocin belonging to a new family of unmodified bacteriocins of gram-positive bacteria. *Applied and Environmental Microbiology*, 73 (9): 2871-2877. doi: 10.1128/aem.02286-06.

Gabrielsen, C., Brede, D. A., Nes, I. F. & Diep, D. B. (2014). Circular Bacteriocins: Biosynthesis and Mode of Action. *Applied and Environmental Microbiology*, 80 (22): 6854-6862. doi: 10.1128/AEM.02284-14.

Galvin, M., Hill, C. & Ross, R. P. (1999). Lacticin 3147 displays activity in buffer against gram-positive bacterial pathogens which appear insensitive in standard plate assays. *Lett Appl Microbiol*, 28 (5): 355-8.

Gong, X., Martin-Visscher, L. A., Nahirney, D., Vederas, J. C. & Duszyk, M. (2009). The circular bacteriocin, carnocyclin A, forms anion-selective channels in lipid bilayers. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1788 (9): 1797-1803. doi: https://doi.org/10.1016/j.bbamem.2009.05.008.

Haldorsen, S. T. (2017). *Screening for antimicrobials against Staphylococcus aureus and Pseudomonas aeruginosa in fermented fruit and vegetables*. 30 ECTS. Ås: Norwegian University of Life Sciences.

Head, S. R., Komori, H. K., LaMere, S. A., Whisenant, T., Van Nieuwerburgh, F., Salomon, D. R. & Ordoukhanian, P. (2014). Library construction for next-generation sequencing: overviews and challenges. *Biotechniques*, 56 (2): 61-4, 66, 68, passim. doi: 10.2144/000114133.

Henderson, J. T., Chopko, A. L. & van Wassenaar, P. D. (1992). Purification and primary structure of pediocin PA-1 produced by Pediococcus acidilactici PAC-1.0. *Archives of Biochemistry and Biophysics*, 295 (1): 5-12. doi: https://doi.org/10.1016/0003-9861(92)90480-K.

Himeno, K., Fujita, K., Zendo, T., Wilaipun, P., Ishibashi, N., Masuda, Y., Yoneyama, F., Leelawatcharamas, V., Nakayama, J. & Sonomoto, K. (2012). Identification of enterocin NKR-5-3C, a novel class IIa bacteriocin produced by a multiple bacteriocin producer, Enterococcus faecium NKR-5-3. *Biosci Biotechnol Biochem*, 76 (6): 1245-7. doi: 10.1271/bbb.120089.

Holo, H., Nilssen, O. & Nes, I. F. (1991). Lactococcin A, a new bacteriocin from Lactococcus lactis subsp. cremoris: isolation and characterization of the protein and its gene. *J Bacteriol*, 173 (12): 3879-87.

Ishibashi, N., Himeno, K., Masuda, Y., Perez, R. H., Iwatani, S., Zendo, T., Wilaipun, P., Leelawatcharamas, V., Nakayama, J. & Sonomoto, K. (2014). Gene Cluster Responsible for Secretion of and Immunity to Multiple Bacteriocins, the NKR-5-3 Enterocins. *Applied and Environmental Microbiology*, 80 (21): 6647-6655. doi: 10.1128/aem.02312-14.

Kaur, K., Tarassova, O., Dangeti, R. V., Azmi, S., Wishart, D., McMullen, L. & Stiles, M. (2016). Characterization of a highly potent antimicrobial peptide microcin N from uropathogenic Escherichia coli. *FEMS Microbiology Letters*, 363 (11): fnw095-fnw095. doi: 10.1093/femsle/fnw095.

Kennedy, S. (2017). *How DNA extraction kits work in the lab*. Available at: https://bitesizebio.com/13516/how-dna-extraction-rna-miniprep-kits-work/ (accessed: April 16, 2018).
Kerr, A. P. (2013). *The Bacteriostatic Spectrum and Inhibitory Mechanism of Glycocin F, a Bacteriocin from Lactococcus plantarum KW30*. Palmerston North, New Zealand: Massey University.
Khan, H., Flint, S. H. & Yu, P. L. (2013). Determination of the mode of action of enterolysin A, produced by Enterococcus faecalis B9510. *J Appl Microbiol*, 115 (2): 484-94. doi: 10.1111/jam.12240.
Kjos, M., Borrero, J., Opsata, M., Birri, D. J., Holo, H., Cintas, L. M., Snipen, L., Hernandez, P. E., Nes, I. F. & Diep, D. B. (2011). Target recognition, resistance, immunity and genome mining of class II bacteriocins from Gram-positive bacteria. *Microbiology*, 157 (Pt 12): 3256-67. doi: 10.1099/mic.0.052571-0.
Latha, S. & Dhanasekaran, D. (2015). Bacteriocin, a natural alternative to synthetic antimicrobial antibiotics. In Dhanasekaran, D., Thajuddin, N. & Panneerselvam, A. (eds) *Antimicrobials: Synthetic and Natural Compounds*: CRC Press.
López-Cuellar, M. d. R., Rodríguez-Hernández, A.-I. & Chavarría-Hernández, N. (2016). LAB bacteriocin applications in the last decade. *Biotechnology & Biotechnological Equipment*, 30 (6): 1039-1050. doi: 10.1080/13102818.2016.1232605.
Maldonado-Barragán, A., Ruiz-Barba, J. L. & Jiménez-Díaz, R. (2009). Knockout of three-component regulatory systems reveals that the apparently constitutive plantaricin-production phenotype shown by Lactobacillus plantarum on solid medium is regulated via quorum sensing. *International Journal of Food Microbiology*, 130 (1): 35-42. doi: https://doi.org/10.1016/j.ijfoodmicro.2008.12.033.
Martens, E. & Demain, A. L. (2017). The antibiotic resistance crisis, with a focus on the United States. *J Antibiot (Tokyo)*, 70 (5): 520-526. doi: 10.1038/ja.2017.30.
Martinez, B., Fernandez, M., Suarez, J. E. & Rodriguez, A. (1999). Synthesis of lactococcin 972, a bacteriocin produced by Lactococcus lactis IPLA 972, depends on the expression of a plasmid-encoded bicistronic operon. *Microbiology*, 145 (Pt 11): 3155-61. doi: 10.1099/00221287-145-11-3155.
Martinez, B., Rodriguez, A. & Suarez, J. E. (2000). Lactococcin 972, a bacteriocin that inhibits septum formation in lactococci. *Microbiology*, 146 (Pt 4): 949-55. doi: 10.1099/00221287-146-4-949.
Martínez, B., Rodriguez, A. & Suárez, E. (2016). Antimicrobial Peptides Produced by Bacteria: The Bacteriocins. In Villa, T. G. & Vinas, M. (eds) *New Weapons to Control Bacterial Growth*, pp. 15-38: Springer International Publishing Switzerland.
Mokoena, M. P. (2017). Lactic Acid Bacteria and Their Bacteriocins: Classification, Biosynthesis and Applications against Uropathogens: A Mini-Review. *Molecules*, 22 (8). doi: 10.3390/molecules22081255.
Molloy, E., Cotter, P., Hill, C., Mitchell, D. & Paul Ross, R. (2011). *Streptolysin S-like virulence factors: The continuing sagA*, vol. 9.
Morse, S. S. (2009). EMERGING INFECTIONS: CONDEMNED TO REPEAT? In Institute of Medicine Forum on Microbial, T. (ed.) *Microbial Evolution and Co-Adaptation: A Tribute to the Life and Scientific Legacies of Joshua Lederberg: Workshop Summary*. Washington (DC): National Academies Press (US).
Nes, I. F., Holo, H., Fimland, G., Hauge, H. H. & Nissen-Meyer, J. (2001). Unmodified Peptide-Bacteriocins (Class II) Produced by Lactic Acid Bacteria. In Dutton, C., Haxwell, M., McArthur, H. & Wax, R. G. (eds) *Peptide Antibiotics: Discovery Modes Of Action And Applications*, pp. 67-68: Taylor & Francis.
Neysens, P., Messens, W. & De Vuyst, L. (2003). Effect of sodium chloride on growth and bacteriocin production by Lactobacillus amylovorus DCE 471. *Int J Food Microbiol*, 88 (1): 29-39.
Nilsen, T., Nes, I. F. & Holo, H. (2003). Enterolysin A, a cell wall-degrading bacteriocin from Enterococcus faecalis LMG 2333. *Applied and Environmental Microbiology*, 69 (5): 2975-2984. doi: 10.1128/aem.69.5.2975-2984.2003.

Nissen-Meyer, J., Holo, H., Håvarstein, L. S., Sletten, K. & Nes, I. F. (1992). A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. *Journal of Bacteriology*, 174 (17): 5686-5692.

Nissen-Meyer, J., Oppegård, C., Rogne, P., Haugen, H. S. & Kristiansen, P. E. (2010). Structure and Mode-of-Action of the Two-Peptide (Class-IIb) Bacteriocins. *Probiotics and Antimicrobial Proteins*, 2 (1): 52-60. doi: 10.1007/s12602-009-9021-z.

Oswald, N. (2014). *The Basics: How Alkaline Lysis Works*. Available at: https://bitesizebio.com/180/the-basics-how-alkaline-lysis-works/ (accessed: April 10, 2018).

Perez, R. H., Zendo, T. & Sonomoto, K. (2014). Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications. *Microbial Cell Factories*, 13 (Suppl 1): S3-S3. doi: 10.1186/1475-2859-13-S1-S3.

Perez, R. H., Ishibashi, N., Inoue, T., Himeno, K., Masuda, Y., Sawa, N., Zendo, T., Wilaipun, P., Leelawatcharamas, V., Nakayama, J., et al. (2016). Functional Analysis of Genes Involved in the Biosynthesis of Enterocin NKR-5-3B, a Novel Circular Bacteriocin. *Journal of Bacteriology*, 198 (2): 291-300. doi: 10.1128/jb.00692-15.

Piper, C., Draper, L., Cotter, P., Paul Ross, R. & Hill, C. (2009). *A comparison of the activities of lacticin 3147 and nisin against drug-resistant Staphylococcus aureus and Enterococcus species*, vol. 64.

Rea, M., Paul Ross, R., Cotter, P. & Hill, C. (2011). Classification of Bacteriocins from Gram-Positive Bacteria. In Drider, D. & Rebuffat, S. (eds) *Prokaryotic Antimicrobial Peptides, From Genes to Applications*, pp. 29-48: Springer.

Salomon, R. A. & Farias, R. N. (1992). Microcin 25, a novel antimicrobial peptide produced by Escherichia coli. *J Bacteriol*, 174 (22): 7428-35.

Sawa, N., Wilaipun, P., Kinoshita, S., Zendo, T., Leelawatcharamas, V., Nakayama, J. & Sonomoto, K. (2012). Isolation and Characterization of Enterocin W, a Novel Two-Peptide Lantibiotic Produced by Enterococcus faecalis NKR-4-1. *Applied and Environmental Microbiology*, 78 (3): 900-903. doi: 10.1128/AEM.06497-11.

Scholz, R., Vater, J., Budiharjo, A., Wang, Z., He, Y., Dietel, K., Schwecke, T., Herfort, S., Lasch, P. & Borriss, R. (2014). Amylocyclicin, a novel circular bacteriocin produced by Bacillus amyloliquefaciens FZB42. *J Bacteriol*, 196 (10): 1842-52. doi: 10.1128/JB.01474-14.

Singh, P. K., Chittpurna, Ashish, Sharma, V., Patil, P. B. & Korpole, S. (2012). Identification, Purification and Characterization of Laterosporulin, a Novel Bacteriocin Produced by Brevibacillus sp. Strain GI-9. *PLoS ONE*, 7 (3): e31498. doi: 10.1371/journal.pone.0031498.

Singhal, N., Kumar, M., Kanaujia, P. K. & Virdi, J. S. (2015). MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. *Front Microbiol*, 6: 791. doi: 10.3389/fmicb.2015.00791.

Stepper, J., Shastri, S., Loo, T. S., Preston, J. C., Novak, P., Man, P., Moore, C. H., Havlíček, V., Patchett, M. L. & Norris, G. E. (2011). Cysteine S-glycosylation, a new post-translational modification found in glycopeptide bacteriocins. *FEBS Letters*, 585 (4): 645-650. doi: https://doi.org/10.1016/j.febslet.2011.01.023.

Swe, P. M., Cook, G. M., Tagg, J. R. & Jack, R. W. (2009). Mode of action of dysgalacticin: a large heat-labile bacteriocin. *Journal of Antimicrobial Chemotherapy*, 63 (4): 679-686. doi: 10.1093/jac/dkn552.

Tanasupawat, S., Sukontasing, S. & Lee, J. S. (2008). Enterococcus thailandicus sp. nov., isolated from fermented sausage ('mum') in Thailand. *Int J Syst Evol Microbiol*, 58 (Pt 7): 1630-4. doi: 10.1099/ijs.0.65535-0.

Tobes, R. & Pareja, E. (2006). Bacterial repetitive extragenic palindromic sequences are DNA targets for Insertion Sequence elements. *BMC Genomics*, 7: 62. doi: 10.1186/1471-2164-7-62.

Turgis, M., Vu, K. D., Millette, M., Dupont, C. & Lacroix, M. (2016). Influence of Environmental Factors on Bacteriocin Production by Human Isolates of Lactococcus lactis MM19 and Pediococcus acidilactici MM33. *Probiotics Antimicrob Proteins*, 8 (1): 53-9. doi: 10.1007/s12602-015-9204-8.

van Belkum, M. J., Martin-Visscher, L. A. & Vederas, J. C. (2011). Structure and genetics of circular bacteriocins. *Trends Microbiol*, 19 (8): 411-8. doi: 10.1016/j.tim.2011.04.004.
Vendrell, D., Balcázar, J. L., Ruiz-Zarzuela, I., de Blas, I., Gironés, O. & Múzquiz, J. L. (2006). Lactococcus garvieae in fish: A review. *Comparative Immunology, Microbiology and Infectious Diseases*, 29 (4): 177-198. doi: https://doi.org/10.1016/j.cimid.2006.06.003.
Ventola, C. L. (2015a). The Antibiotic Resistance Crisis: Part 1: Causes and Threats. *Pharmacy and Therapeutics*, 40 (4): 277-283.
Ventola, C. L. (2015b). The Antibiotic Resistance Crisis: Part 2: Management Strategies and New Agents. *Pharmacy and Therapeutics*, 40 (5): 344-352.
Vera Pingitore, E., Salvucci, E., Sesma, F. & Nader-Macías, M. (2007). Different strategies for purification of antimicrobial peptides from Lactic Acid Bacteria (LAB). In Méndez-Vilas, A. (ed.) vol. 2 *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*.
Versalovic, J., Schneider, M., De Bruijn, F. J. & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology*, 5 (1): 25-40.
Wan, X. (2017). *Leuconostoc bacteriocins and their application in genome editing*. Doctoral dissertation. Finland: University of Helsinki.
Wooley, R. E., Gibbs, P. S. & Shotts, E. B. (1999). Inhibition of Salmonella typhimurium in the Chicken Intestinal Tract by a Transformed Avirulent Avian Escherichia coli. *Avian Diseases*, 43 (2): 245-250. doi: 10.2307/1592614.
Yang, R. (1994). Factors influencing production of bacteriocins by lactic acid bacteria. *Food Microbiology*, 11: 281-291. doi: 10.1006/fmic.1994.1032.
Yang, S.-C., Lin, C.-H., Sung, C. T. & Fang, J.-Y. (2014). Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Frontiers in Microbiology*, 5: 241. doi: 10.3389/fmicb.2014.00241.
Yoneyama, H. & Katsumata, R. (2006). Antibiotic resistance in bacteria and its future for novel antibiotic development. *Biosci Biotechnol Biochem*, 70 (5): 1060-75. doi: 10.1271/bbb.70.1060.
Zendo, T., Koga, S., Shigeri, Y., Nakayama, J. & Sonomoto, K. (2006). Lactococcin Q, a Novel Two-Peptide Bacteriocin Produced by Lactococcus lactis QU 4. *Applied and Environmental Microbiology*, 72 (5): 3383-3389. doi: 10.1128/AEM.72.5.3383-3389.2006.
Zendo, T. (2011). Classification and diversity of bacteriocins. In Sonomoto, K. & Yokota, A. (eds) *Lactic Acid Bacteria and Bifidobacteria: Current Progress in Advanced Research*, pp. 160-163: Caister Academic Press.

Appendix

Table 1. The source of the 50 samples

SAMPLE	SOURCE	SAMPLE	SOURCE
1	Green grapes	26	Banana
2	Blue grapes	27	Kelek/Turkish cucumber
3	Orange water melon	28	Romanesco broccoli
4	Small cucumber	29	Sugar peas
5	Sweet cherries	30	Aristo
6	Avocado	31	Chinese cabbage
7	Pineapple	32	Quince (Kvede)
8	Purple aubergine	33	Fichianindia
9	Apricot	34	Rambutan
10	Raspberries	35	Eddo/Taro
11	Blueberries	36	Fig
12	Tomatoes	37	Green, small mango
13	Mango	38	Sweet potato
14	Blue plum	39	Lychee
15	Ladyfinger/Okra	40	Sharon
16	Dates	41	Aubergine
17	Passion fruit	42	Large chili
18	Strawberries	43	Kiwi
19	Plums	44	Red onion
20	White aubergine	45	Blackberry
21	Physalis	46	Rotten apple from the garden
22	Pear	47	Small, green chili
23	Karela/Balsam pear	48	Dragon fruit (Thanh long)
24	Taro/Eddo roots	49	Jackfruit
25	Chayote/Chow chow	50	Longgong

Table 2. Results of the antimicrobial spectrum test against the 54 indicators. The isolates in the table were tested on MRS plates. The Asterix * indicates the presence of resistant cells inside the zones

			BACTERIA ISOLATES GROWN ON MRS PLATES							
species			*E. faecium*		*E.thailandicus*	*L. mesenteroides*	*L. garvieae*	*L. lactis*	*L. lactis B1627*	
Sample number			28 +		35 +	34	23 +	6	Control	
			isolate 1	isolate 3	isolate 1	isolate 1	isolate 5	isolate 1		
		INDICATORS								
	strain nr	species								
1	LMGT2805	*Bacillus cereus*	0	0	0.5	3	0	1	1	
2	LMGT 2711	*Bacillus cereus ATCC 9139 B*	0	0	0	1	0	1	1	
3	LMGT 2731	*Bacillus cereus 1230, Granum 11-91*	0	0	0.5	2	0	1	1	
4	LMGT 2736	*B.cereus ATCC 2 (Matforsk)*	0	0	0	0	0	1	1	
9	LMGT 2738	*Carnobacterium divergens NCDO 2306*	0	0.5	2	0	2	1	1	
10	LMGT2332	*Carnobacterium piscicola*	0.5*	1	0.5*	1	1	2	2	
11	LMGT3465	*Enterococcus avium*	0.5	0.5	1	0.5	0.5	1	1	
12	LMGT2333	*Enterococccus faecalis*	0.5	1	1	0	0	0.5	0.5	
13	LMG3088	*Enterococcus faecalis*	0.5	1	1	0	1	0.5	0.5	
14	LMGT 3330	*E. faecalis 158B*	0	1	0	0	0	0	0	
15	LMGT 3331	*E. faecalis 111A*	0	0	0	0	0	0	0	
16	LMGT 3332	*E. faecalis 29C*	0	0	0	0	0	0	0	
17	LMGT 2763	*E. faecium*	0	0	0	0	0.5	1	1	
18	LMGT2772	*E.faecium*	0	0.5	0.5	0.5	0.5	1	1	
19	LMGT2783	*E. faecium*	0	0.5	0	0	0	0.5	0.5	
20	LMGT2876	*E. faecium*	0	0	0	0	0	1	1	
21	LMGT2353	*Lactobacillus curvatus*	0	0.5*	1	1	0	1	1	
22	LMGT2355	*L. curvatus*	1*	0.5	2*	1	0.5	2*	2	
23	LMGT2003	*L. plantarum*	0	1	1	0.5	0	1	1	
24	LMGT2352	*L. plantarum*	0.5	0.5	2	0	0	3	3	
25	LMGT3125	*L. plantarum*	0	0	1	0	0	2	2	
26	LMGT2361	*L. sakei*	0.5	0.5	1	1	0	2	2	
27	LMGT2380	*L. sakei*	0.5	0.5	1	1	0	3	3	
28	LMGT2787	*L. salivarius*	1	1	2	0.5	2	2	2	
29	LMGT3390	*Lactococcus garvieae*	0.5	0.5	1	0	1	1	1	
30	IL1403	*L. lactis*	0	0.5	1	1	0.5	2	2	
31	LMGT2081	*L. lactis*	0	0.5	0	1	0	0	0	
32	LMGT2386	*Leuconostoc gelidium*	0	0	0	0	0	0	0	
33	LMGT2710	*Listeria innocua*	0	0.5	0	0.5	0.5	2	2	
34	LMGT2785	*L. innocua*	0	1	0	0	0	1	1	
35	LMGT2813	*L. ivanovii*	0	0.5	1	1	2	2	2	
36	LMGT2604	*L. monocytogenes*	0	0.5	1	0	0	0	0	
37	LMGT2650	*L. monocytogenes*	0	0	0.5	1	0	0	0	
38	LMGT2651	*L. monocytogenes*	0	0.5	1	0.5	1	2	2	
39	LMGT2652	*L. monocytogenes*	0	0.5	1	1	1	2	2	
40	LMGT2653	*L.monocytogenes*	0.5	0.5	1	1	1	2	2	
41	LMGT2002	*Pediococcus acidilactici*	0	0	1	1	0	2	2	
42	LMGT2001	*P. pentosaceus*	0	2	0.5	2	0.5	3	3	
43	LMGT2366	*P. pentosaceus*	0	0.5	1	0.5	0	2	2	
44	LMGT3022	*Staphylococcus aureus*	0	0	1	0	0	1	1	
45	LMGT3023	*S. aureus*	0	0	0	1	0.5	1	1	
46	LMGT3242	*S. aureus*	0.5	0.5	0.5	2	0.5	1	1	
47	LMGT3262	*S. aureus*	0.5	0.5	0.5	1	0.5	1	1	
48	LMGT3263	*S. aureus*	0.5	0.5	0.5	1	0.5	1	1	
49	LMGT3264	*S. aureus*	0	0	0	1	0.5	1	1	
50	LMGT3265	*S. aureus*	0	0	0	1	0.5	1	1	
58	LMGT 3347	*Strep agalactiae Val 373706*	0	0	0	0	0.5	0	0	
		Dr Thomas Fielder U i Rostock								
59	LMGT 3890	*Str. dysgalactiae*	0	0	0	1	1	1	1	
60	LMGT 3899	*Str. dysgalactiae*	0	0	0.5	0.5	0	0.5	0.5	
61	LMG3555	*Streptococcus thermophilus sfi13*	1	1	1	1	1	2	2	
		Streptococcus pneumoniae D39	0.5	2	2	0	0	N/A	N/A	
		L. garvieae B1515 (garvicin ML producer)	1	1	2	0.5	1	N/A	N/A	
		L. garvieae B1642 (garvicin KS producer)	0.5	0	1	0	1	N/A	N/A	
		L. garvieae B1680	1	0.5	1	0.5	1	N/A	N/A	

Table 3. Results of the antimicrobial spectrum test against the 54 indicators. The following isolate were tested on BHI plates.

			BACTERIA ISOLATES GROWN ON BHI PLATES					
Species			*E. faecalis*		*L. lactis*			
Sample number			15		42	48	29	control
			isolate 1	isolate 2	Isolate 1	isolate 1	isolate 1	strain B1627
		INDICATORS						
	strain nr	species						
1	LMGT2805	*Bacillus cereus*	0	0	1	1	1	1
2	LMGT 2711	*Bacillus cereus ATCC 9139 B*	0	0	1	1	1	1
3	LMGT 2731	*Bacillus cereus 1230, Granum 11-91*	0	0	1*	1*	1*	1*
4	LMGT 2736	*B.cereus ATCC 2 (Matforsk)*	0	0	1	1	1	1
9	LMGT 2738	*Carnobacterium divergens NCDO 2306*	0.5	1	1	1	1	1
10	LMGT2332	*Carnobacterium piscicola*	0	0	0.5	0.5	0.5	0.5
11	LMGT3465	*Enterococcus avium*	0*	1	0.5	0.5	0.5	0.5
12	LMGT2333	*Enterococccus faecalis*	0	0	0.5	0.5	0.5	0.5
13	LMG3088	*Enterococcus faecalis*	0.5	0.5	0.5	0.5	0.5	0.5
14	LMGT 3330	*E. faecalis 158B*	0	0	0	0	0	0
15	LMGT 3331	*E. faecalis 111A*	0	0	0.5*	0.5*	0.5*	0.5*
16	LMGT 3332	*E. faecalis 29C*	0.5	0.5	0.5	0.5	0.5	0.5
17	LMGT 2763	*E. faecium*	0.5	1	1	1	1	1
18	LMGT2772	*E.faecium*	0.5	1	1	1	1	1
19	LMGT2783	*E. faecium*	0.5	1	1	1	1	1
20	LMGT2876	*E. faecium*	0.5	1	0.5	0.5	0.5	0.5
21	LMGT2353	*Lactobacillus curvatus*	1	2	1	1	1	1
22	LMGT2355	*L. curvatus*	0.5	1	0.5	0.5	0.5	0.5
23	LMGT2003	*L. plantarum*	0.5	0.5	0.5	0.5	0.5	0.5
24	LMGT2352	*L. plantarum*	1	0	3	3	3	3
25	LMGT3125	*L. plantarum*	1	0	2	2	2	2
26	LMGT2361	*L. sakei*	1	1	2	2	2	2
27	LMGT2380	*L. sakei*	2	3	3	3	3	3
28	LMGT2787	*L. salivarius*	0	0	0	0	0	0
29	LMGT3390	*Lactococcus garvieae*	0	0	1	1	1	1
30	IL1403	*L. lactis*	0	0	0	0	0	0
31	LMGT2081	*L. lactis*	0	1	0	0	0	0
32	LMGT2386	*Leuconostoc gelidium*	0	1*	0	0	0	0
33	LMGT2710	*Listeria innocua*	0.5	0.5	0.5	0.5	0.5	0.5
34	LMGT2785	*L. innocua*	0.5	1	1	1	1	1
35	LMGT2813	*L. ivanovii*	1	1	2	2	2	2
36	LMGT2604	*L. monocytogenes*	0	0.5	1	1	1	0.5
37	LMGT2650	*L. monocytogenes*	0	0	0	0	0	0
38	LMGT2651	*L. monocytogenes*	1	1	1	1	1	1
39	LMGT2652	*L. monocytogenes*	1	1	1	1	1	1
40	LMGT2653	*L.monocytogenes*	1	1	0.5	0.5	0.5	0.5
41	LMGT2002	*Pediococcus acidilactici*	0.5*	2	2	2	2	2
42	LMGT2001	*P. pentosaceus*	1	2	3	3	3	3
43	LMGT2366	*P. pentosaceus*	1*	1	2	2	2	2
44	LMGT3022	*Staphylococcus aureus*	1	2	1	1	1	1
45	LMGT3023	*S. aureus*	0	0	1	1	1	1
46	LMGT3242	*S. aureus*	0	0	0.5	0.5	0.5	0.5
47	LMGT3262	*S. aureus*	0.5	0	0.5	0.5	0.5	0.5
48	LMGT3263	*S. aureus*	0	0	1	1	1	1
49	LMGT3264	*S. aureus*	0	0	0.5	0.5	0.5	0.5
50	LMGT3265	*S. aureus*	0	0	1	1	1	1
58	LMGT 3347	*Strep agalactiae Val 373706*	0	0	0	0	0	0
		Dr Thomas Fielder U i Rostock						
59	LMGT 3890	*Str. dysgalactiae*	0*	0	0	0	0	0
60	LMGT 3899	*Str. dysgalactiae*	0.5	0	1	1	1	1
61	LMG3555	*Streptococcus thermophilus sfi13*	1	3	1	1	1	1
		Streptococcus pneumoniae D39	2	3	N/A	N/A	N/A	N/A
		L. garvieae B1515 (garvicin ML producer)	1	0.5	N/A	N/A	N/A	N/A
		L. garvieae B1642 (garvicin KS producer)	0	0	N/A	N/A	N/A	N/A
		L. garvieae B1680	0.5	0.5	N/A	N/A	N/A	N/A

Table 4. Sequence alignment for the candidate bacteriocins from each sequenced genome

E. faecalis isolate 1

Microcin-24 [Yersinia ruckeri ATCC 29473]

Sequence ID: EEP99451.1

Score	Expect	Method	Identities	Positives	Gaps
204 bits(518)	2E-66	Compositional matrix adjust.	102/102(100%)	102/102(100%)	0/102(0%)

```
Query  1    MIKLNNEEMSCVYGSVDNRQVIKDILIDSVLGAGFGAPGGPPGMLLGAGLGASQSVIHSA  60
            MIKLNNEEMSCVYGSVDNRQVIKDILIDSVLGAGFGAPGGPPGMLLGAGLGASQSVIHSA
Sbjct  1    MIKLNNEEMSCVYGSVDNRQVIKDILIDSVLGAGFGAPGGPPGMLLGAGLGASQSVIHSA  60

Query  61   INHGPVDVKIPTVPMGPIWNGSGVNIMKNTWVPGFGSKVNMY  102
            INHGPVDVKIPTVPMGPIWNGSGVNIMKNTWVPGFGSKVNMY
Sbjct  61   INHGPVDVKIPTVPMGPIWNGSGVNIMKNTWVPGFGSKVNMY  102
```

E. faecalis isolate 2

enterocin W alfa [Enterococcus faecalis]

Sequence ID: BAL50001.1

Score	Expect	Method	Identities	Positives	Gaps
123 bits(309)	7E-36	Compositional matrix adjust.	59/60(98%)	60/60(100%)	0/60(0%)

```
Query  1    MKKEELVGLAKEDFLNVICENDNKLENSGAKCPWWNLSCHLGNDGKICTYSHECTAGCNA  60
            MKKEELVG+AKEDFLNVICENDNKLENSGAKCPWWNLSCHLGNDGKICTYSHECTAGCNA
Sbjct  1    MKKEELVGMAKEDFLNVICENDNKLENSGAKCPWWNLSCHLGNDGKICTYSHECTAGCNA  60
```

enterocin W beta [Enterococcus faecalis]

Sequence ID: BAL50002.1

Score	Expect	Method	Identities	Positives	Gaps
124 bits(311)	4E-36	Compositional matrix adjust.	61/61(100%)	61/61(100%)	0/61(0%)

```
Query  1    MTELNKRLQLKRDVSTENSLKKISNTDETHGGVTTSIPCTVMVSAAVCPTLVCSNKCGGR  60
            MTELNKRLQLKRDVSTENSLKKISNTDETHGGVTTSIPCTVMVSAAVCPTLVCSNKCGGR
Sbjct  1    MTELNKRLQLKRDVSTENSLKKISNTDETHGGVTTSIPCTVMVSAAVCPTLVCSNKCGGR  60

Query  61   G  61
            G
Sbjct  61   G  61
```

enterolysin A [Enterococcus faecalis]

Sequence ID: AAG29099.1

Score	Expect	Method	Identities	Positives	Gaps
699 bits(1804)	0.0	Compositional matrix adjust.	343/343(100%)	343/343(100%)	0/343(0%)

```
Query  1    MKNILLSILGVLSIVVSLAFSSYSVNAASNEWSWPLGKPYAGRYEEGQQFGNTAFNRGGT  60
            MKNILLSILGVLSIVVSLAFSSYSVNAASNEWSWPLGKPYAGRYEEGQQFGNTAFNRGGT
Sbjct  1    MKNILLSILGVLSIVVSLAFSSYSVNAASNEWSWPLGKPYAGRYEEGQQFGNTAFNRGGT  60

Query  61   YFHDGFDFGSAIYGNGSVYAVHDGKILYAGWDPVGGGSLGAFIVLQAGNTNVIYQEFSRN  120
            YFHDGFDFGSAIYGNGSVYAVHDGKILYAGWDPVGGGSLGAFIVLQAGNTNVIYQEFSRN
Sbjct  61   YFHDGFDFGSAIYGNGSVYAVHDGKILYAGWDPVGGGSLGAFIVLQAGNTNVIYQEFSRN  120

Query  121  VGDIKVSTGQTVKKGQLIGKFTSSHLHLGMTKKEWRSAHSSWNKDDGTWFNPIPILQGGS  180
            VGDIKVSTGQTVKKGQLIGKFTSSHLHLGMTKKEWRSAHSSWNKDDGTWFNPIPILQGGS
Sbjct  121  VGDIKVSTGQTVKKGQLIGKFTSSHLHLGMTKKEWRSAHSSWNKDDGTWFNPIPILQGGS  180

Query  181  TPTPPNPGPKNFTTNVRYGLRVLGGSWLPEVTNFNNTNDGFAGYPNRQHDMLYIKVDKGQ  240
            TPTPPNPGPKNFTTNVRYGLRVLGGSWLPEVTNFNNTNDGFAGYPNRQHDMLYIKVDKGQ
Sbjct  181  TPTPPNPGPKNFTTNVRYGLRVLGGSWLPEVTNFNNTNDGFAGYPNRQHDMLYIKVDKGQ  240

Query  241  MKYRVHTAQSGWLPWVSKGDKSDTVNGAAGMPGQAIDGVQLNYITPKGEKLSQAYYRSQT  300
            MKYRVHTAQSGWLPWVSKGDKSDTVNGAAGMPGQAIDGVQLNYITPKGEKLSQAYYRSQT
Sbjct  241  MKYRVHTAQSGWLPWVSKGDKSDTVNGAAGMPGQAIDGVQLNYITPKGEKLSQAYYRSQT  300

Query  301  TKRSGWLKVSADNGSIPGLDSYAGIFGEPLDRLQIGISQSNPF  343
            TKRSGWLKVSADNGSIPGLDSYAGIFGEPLDRLQIGISQSNPF
Sbjct  301  TKRSGWLKVSADNGSIPGLDSYAGIFGEPLDRLQIGISQSNPF  343
```

E. faecium isolate 1

peptidase M23 [Enterococcus faecium]

Sequence ID: WP_086327814.1

Score	Expect	Method	Identities	Positives	Gaps
726 bits(1875)	0.0	Compositional matrix adjust.	358/360(99%)	358/360(99%)	0/360(0%)

```
Query  1    MMGVLFLFFGVDDSDTSGSTAGGTEFNGVYTEDLPSYPEIKGVGNVPDEIAQLAVGSAVK  60
            MMGVLFLFFGVDDSDTSGSTAGGTEFNGVYTEDLPSYPEIKGVGNVPDEIAQLAVGSAVK
Sbjct  20   MMGVLFLFFGVDDSDTSGSTAGGTEFNGVYTEDLPSYPEIKGVGNVPDEIAQLAVGSAVK  79

Query  61   YHLLPSVIISQWAYESEWGHSASAKNDNNFFGITWFEGCPFPKGTARGVGGSEGGNYMKF  120
            YHLLPSVIISQWAYESEWGHSASAKNDNNFFGITWFEGCPFPKGTARGVGGSEGGNYMKF
Sbjct  80   YHLLPSVIISQWAYESEWGHSASAKNDNNFFGITWFEGCPFPKGTARGVGGSEGGNYMKF  139

Query  121  PNKKSAFSYYGYMVAFQTNFNACVGNKSPEQCLLTLGRGGYAAAGISINSPYFTGCMSII  180
            PNKKSAFSYYGYMVAFQTNFNACVGNKSPEQCLLTLGRGGYAAAGISINSPYFTGCMSII
Sbjct  140  PNKKSAFSYYGYMVAFQTNFNACVGNKSPEQCLLTLGRGGYAAAGISINSPYFTGCMSII  199

Query  181  KSNNLTQYDDFAIKNWKDFGGNTGGSVGGGWGWPFPEVGQGSFAGGQLFGKNPGGEFREN  240
            KSNNLTQYDDFAIKNWKDFGGNTGGSVGGGWGWPF EVGQGSFAGGQLFGKNPGGEFREN
Sbjct  200  KSNNLTQYDDFAIKNWKDFGGNTGGSVGGGWGWPFLEVGQGSFAGGQLFGKNPGGEFREN  259

Query  241  GWHDGLDFGSVDHPGSEIHAVHGGTVTYVGNPNIGGLGACVIVINDSGLNMVYQEFATST  300
            GWHDGLDFGSVDHPGSEIHAVHGGTVTYVGNPNIGGLGACVIVINDSGLNMVYQEFATST
Sbjct  260  GWHDGLDFGSVDHPGSEIHAVHGGTVTYVGNPNIGGLGACVIVINDSGLNMVYQEFATST  319

Query  301  SNAKVKVGDKVKLGDVIGIRDTEHLHLGITKKDWLQAESSAFTDDGTWLDPLKIITTGKY  360
            SNAKVKVGDKVKLGDVIGIRDTEHLHLGITKKDWLQAESSAFTDDGTWLDPLKIIT GKY
Sbjct  320  SNAKVKVGDKVKLGDVIGIRDTEHLHLGITKKDWLQAESSAFTDDGTWLDPLKIITPGKY  379
```

enterocin NKR-5-3B [Enterococcus faecium]

Sequence ID: BAU40203.1

Score	Expect	Method	Identities	Positives	Gaps
154 bits(390)	2E-47	Compositional matrix adjust.	83/87(95%)	86/87(98%)	0/87(0%)

```
Query  1    MKKNLLLVLPILGIVGLFVGAPMLTANLGISSYAAKKVIDIINTGSAVATIISI VAAVVG  60
            MKKNLLLVLPI+GIVGLFVGAPMLTANLGISSYAAKKVIDIINTGSAVATII++V  AVVG
Sbjct  1    MKKNLLLVLPIVGIVGLFVGAPMLTANLGISSYAAKKVIDIINTGSAVATIIALVTAVVG  60

Query  61   GGLITAGIVATAKSLIKKYGAKYAAAW  87
            GGLITAGIVATAKSLIKKYGAKYAAAW
Sbjct  61   GGLITAGIVATAKSLIKKYGAKYAAAW  87
```

E. thailandicus

thaiocin 1, circularin A/uberolysin family circular bacteriocin-like protein [Enterococcus thailandicus]

Sequence ID: ASZ08576.1

Score	Expect	Method	Identities	Positives	Gaps
160 bits(404)	2E-49	Compositional matrix adjust.	87/87(100%)	87/87(100%)	0/87(0%)

```
Query  1   MKKNLLLVLPIVGIVGLFVGAPMLTANLGISSYAAKKVIDIINTGSAVATIIALVTAVVG  60
           MKKNLLLVLPIVGIVGLFVGAPMLTANLGISSYAAKKVIDIINTGSAVATIIALVTAVVG
Sbjct  1   MKKNLLLVLPIVGIVGLFVGAPMLTANLGISSYAAKKVIDIINTGSAVATIIALVTAVVG  60

Query  61  GGLITAGIVATAKSLIKKYGAKYAAAW  87
           GGLITAGIVATAKSLIKKYGAKYAAAW
Sbjct  61  GGLITAGIVATAKSLIKKYGAKYAAAW  87
```

L. mesenteroides

enterocin L50 family leaderless bacteriocin [Enterococcus faecium]

Sequence ID: WP_104777072.1

Score	Expect	Method	Identities	Positives	Gaps
59.3 bits(142)	7E-11	Compositional matrix adjust.	28/43(65%)	35/43(81%)	4/43(9%)

```
Query  1   MGAVARLVLEFG----AKYYKVIMRLIGEGWSVDQIEKYLKRH  39
           MGA+A+LV +FG        KYYK  IM+  IGEGW++DQIEK+LKRH
Sbjct  1   MGAIAKLVAKFGWPIVKKYYKQIMQFIGEGWTIDQIEKWLKRH  43
```

L. garvieae isolate 5

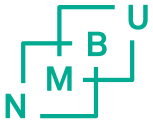
Prepeptide GarQ (garQ) [Lactococcus garvieae IPLA 31405]

Sequence ID: EIT67556.1

Score	Expect	Method	Identities	Positives	Gaps
145 bits(366)	3E-44	Compositional matrix adjust.	70/70(100%)	70/70(100%)	0/70(0%)

```
Query  1   MENNNYTVLSDEELQKIDGGEYHLMNGANGYLTRVNGKYVYRVTKDPVSAVFGVISNGWG  60
           MENNNYTVLSDEELQKIDGGEYHLMNGANGYLTRVNGKYVYRVTKDPVSAVFGVISNGWG
Sbjct  1   MENNNYTVLSDEELQKIDGGEYHLMNGANGYLTRVNGKYVYRVTKDPVSAVFGVISNGWG  60

Query  61  SAGAGFGPQH  70
           SAGAGFGPQH
Sbjct  61  SAGAGFGPQH  70
```



Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway