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Screening for antimicrobials against *Staphylococcus aureus* and *Pseudomonas aeruginosa* in fermented fruit and vegetables

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Abstract

Antibiotics have since the first discovery been the ultimate weapon against pathogenic bacteria. Even though antibiotic resistance is an old phenomenon the emergence of widespread resistance has become a big problem in modern medicine. One possible solution to this problem is a group of antimicrobial peptides, called bacteriocins. These peptides have different modes of action than traditional antibiotics, thus they can be used to kill antibiotic resistant pathogens. It is therefore believed that bacteriocins will be an important part of the pharmaceutical industry, and they are in fact already important in food preservation.

The main aim of this study was to search for bacteria producing bacteriocins/antimicrobials capable of inhibiting *Staphylococcus aureus* and/or *Pseudomonas aeruginosa* in fermented fruit and vegetable samples. This was done using a dilution-based screening method where bacterial colonies showing inhibition of the indicator bacterium was chosen for characterization; spot-on-lawn inhibition assay, fingerprinting (rep-PCR and pulsed field gel electrophoresis (PFGE)), sequencing and fermentation profiling. From screening a total of 44 isolates inhibiting *S. aureus* and 32 isolates inhibiting *P. aeruginosa* were obtained. All bacteria from the *S. aureus* screening showed to be producers of the bacteriocin nisin Z, with some differences in genetic- and fermentation profile between the producers. The bacteria found from screening against *P. aeruginosa* showed different inhibition on MRS and BHI agar, in addition to having a quorum sensing like pattern of inhibition. Further studies have to be done in order to characterize these isolates.

Sammendrag

Antibiotika har siden det ble oppdaget vært et av hovedvirkemidlene mot patogene bakterier. Selv om antibiotikaresistens er et gammelt fenomen har det, etter at antibiotika ble tatt i bruk i stor skala, utviklet seg stadig mer resistens hos bakteriene, noe som er et stort problem innen moderne medisin. En mulig løsning på dette problemet er en gruppe antimikrobielle peptider kalt bakteriosiner. Disse peptidene har andre virkningsmekanismer enn tradisjonell antibiotika, noe som gjør det mulig å bruke dem til å drepe antibiotikaresistente patogener. Det er derfor mulig at bakteriosiner vil bli en viktig del av legemiddelindustrien, og de er allerede en viktig del av preservering av mat og fôr.

Hovedmålet med denne oppgaven var å lete etter bakterier med produksjon av bakteriosiner/antimikrobielle stoffer som hemmet indikatorene *Staphylococcus aureus* og/eller *Pseudomonas aeruginosa* i prøver fra fermentert frukt og grønnsaker. Dette ble gjort ved å bruke en fortynnings-basert fremgangsmåte der bakteriekolonier med hemming av indikatorene ble plukket og videre karakterisert ved bruk av «spot-on-lawn» analyse, fingerprinting (rep-PCR og pulsfelt gelelektroforese (PFGE)), sekvensering og fermenteringsprofilering. Fra screening ble det funnet 44 isolater som hemmet *S. aureus* og 32 isolater som hemmet *P. aeruginosa*. Alle bakteriene funnet i screening mot *S. aureus* viste seg å være produsenter av bakteriosinet nisin Z, med noe variasjon i genetisk- og fermenterings-profil hos produsentene. Bakteriene funnet mot *P. aeruginosa* viste ulik hemming på MRS og BHI medium, samt et «quorum sensing»-liknende inhibisjonsmønster. Videre undersøkelser er nødvendig for karakterisering av disse isolatene.

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Appendix i

1 Introduction

Since the accidental discovery of the first antibiotic, penicillin, done by Alexander Fleming (Fleming 1929), we have discovered a large number of novel groups of antibiotics. In general, traditional antibiotics can be divided into groups based on what kind of target they have, and whether they kill or inhibit microorganisms (bactericidal and bacteriostatic, respectively). The main targets are molecules involved in cell wall synthesis, protein synthesis, and DNA- and RNA synthesis (Kohanski et al. 2010). We have for quite some time had the ultimate weapon against bacteria, but the emergence of antibiotic resistance has made the need for other alternatives essential.

Antibiotic resistance genes were already common before we started to use antibiotics in medicine. Antimicrobials are naturally produced by many microorganisms in the environment, thus making the ability to be resistant an advantage for growth. With increased use of antibiotics the evolutionary pressure has increased enormously, leading to faster gain of resistance (Blair et al. 2014). This forces the development of new tools against the bacteria. However, this is going very slow on the traditional antibiotics front with no novel families to compensate for the resistance to existing antibiotics (Cotter et al. 2013).

1.1 Antimicrobial peptides

The known antimicrobials can be divided into two main groups based on how they are produced; traditional antibiotics and antimicrobial peptides. Traditional antibiotics are not encoded by a specific gene, but rather produced by a multi-enzyme complex, while the other group consists of the ribosomally synthesized peptides which are encoded by a specific (structural) gene, thus only containing proteinogenic amino acids. Some members in this group also undergo post-translational modifications (PTMs) (Jack & Jung 1998). This group contains the antimicrobial peptides (AMPs), including the bacteriocins, which was the main focus of this study.

The AMPs are small peptides with length varying from five to over a hundred amino acids, and since the discovery of the first AMPs by Dubos (1939) over 5,000 types of AMPs have been found or synthesized, originating from both prokaryote and eukaryote organisms (Bahar & Ren 2013). In animals, AMPs are found in tissues and organs exposed to airborne pathogens, and they are believed to be a part of the innate immune system that protects against bacteria, fungi and viruses (Bahar & Ren 2013).

In this study, the main focus will be on the AMPs called bacteriocins, which are produced by both Gram-positive and Gram-negative bacteria (Zacharof & Lovitt 2012). Most of the bacteriocins found in Gram-negative bacteria have been isolated from *Escherichia coli* and other Enterobacteria, often referred to as microcins or colicins. Compared to bacteriocins from Gram-positive bacteria, these bacteriocins have narrower inhibition spectra and are usually only active against other Gram-negative bacteria (Hassan et al. 2012; Nes et al. 2007). The ability to produce bacteriocins is an advantage for the producer because the produced bacteriocin inhibits the growth of other bacteria, reducing the competition. In contrast to traditional antibiotics, the AMPs only inhibits the same or closely related species (Reeves 1965).

Bacteriocins produced by Gram-positive bacteria are of great interest for researchers because they are produced by useful lactic acid bacteria (LAB) in addition to generally having a wider inhibition spectrum than bacteriocins from Gram-negative bacteria. Bacteriocins produced by LAB are also generally regarded as safe (GRAS), since they can be found or used in fermented food and feed products like cheese and yoghurt in addition to being non-toxic to eukaryotic cells (Nes et al. 2007).

1.2 Classification of bacteriocins from LAB

Bacteriocins found in LAB can be grouped into different classes based on different criteria such as producer organism, molecular weight, PTMs, and biological activity (Gharsallaoui et al. 2016). There has for a long time been discussed how this grouping and classification system should be, with constant changes as the research field develops. This leads to some compounds being given more than one name (e.g. thiolbiotics and lantibiotics being the same group) (Sahoo et al. 2016). A majority of the classification systems used, and suggested, are based on the first classification of LAB bacteriocins done by Klaenhammer (1993), dividing the bacteriocins into four distinct classes with several subclasses. One of the most recent attempts to classify the LAB bacteriocins is proposed by Alvarez-Sieiro et al. (2016), and consists of three main classes with several subclasses made to fit the recent discoveries made in the field.

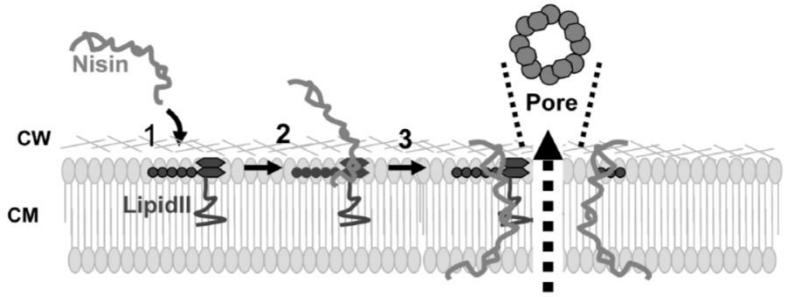
The grouping explained in this paper is based on the work done by Nes et al. (2007) and consists of three classes (**Table 1-1).** There has previously been a fourth class (class III) which contained the large heat-labile bacteriocins, but several of the proteins in this class have

enzymatic activity that targets the cell wall, making them different from bacteriocins. Due to this property, these proteins have been named bacteriolysins (Nes et al. 2007).

Table 1-1 Three classes of bacteriocins with subclasses, brief description and examples. Based on the table from Nes et al. (2007).

Class	Subclass	Description	Example
Class I		Small post-translationally modified peptides (lantibiotics)	
	Type A	Elongated, positively charged (helix-like)	Nisin
	Type B	Globular, anionic or non-charged	Mutacin II
Class II		Non-modified, non-lantibiotic, heat-stable peptides	
	Class IIa	Pediocin-like and strong antilisterial bacteriocins	Pediocin PA-1
	Class IIb	Two-peptide bacteriocins	Plantaricin EF
	Class IIc	Non-pediocin-like, one peptide bacteriocins	Lactococcin A
	Class IId	Leaderless bacteriocins	Lacticin Q
	Class IIe	Peptide bacteriocins formed by specific degradation of proteins	Closticin 574
Class IV		Circular bacteriocins	Enterocin AS-48

Class I contains the small (2-4 kDa), membrane active peptides called lantibiotics. Lantibiotics undergo several PTMs during their biosynthesis, and are characterized by the unusual amino acids lanthionine or methyllanthionine in addition to several other modified amino acids (Klaenhammer 1993; Zacharof & Lovitt 2012). This class is currently divided into two subclasses, type A and type B. Type A consists of linear, elongated, positively charged lantibiotics, while type B comprise the globular and non-charged molecules. Type A lantibiotics, like nisin, inhibit and kill cells by permeabilizing the cell membrane of Gram-positive bacteria by creating pores in the membrane. This leads to loss of membrane potential due to leakage of low-molecular components (**Figure 1-1**). Gram-negative bacteria have an impermeable outer membrane that protects them against the effect of these bacteriocins (Gharsallaoui et al. 2016). Type B lantibiotics, being non-charged, doesn't lead to pore formation of the cell membrane, but rather inhibition of the cell wall synthesis (Zacharof & Lovitt 2012).

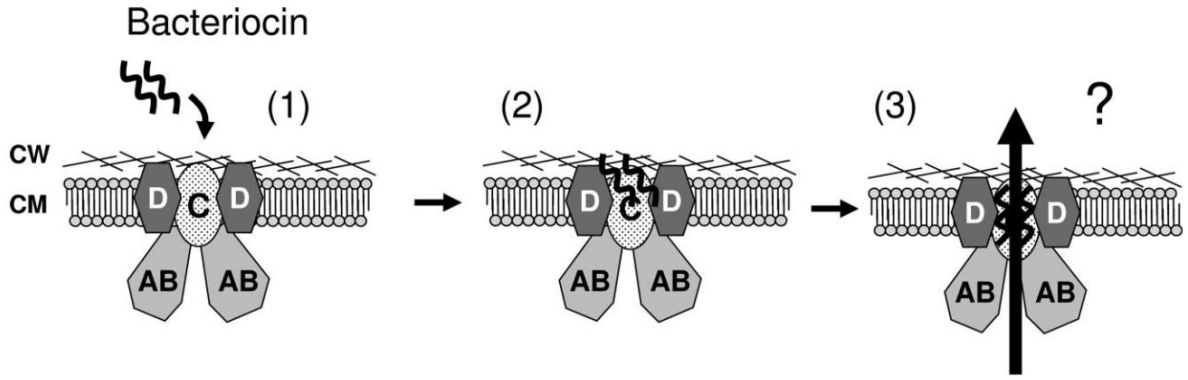


Source: Nes et al. (2007)

Figure 1-1 Illustration showing how nisin targets and binds to a docking molecule (lipid II) in the cell wall (CW) and cytoplasm membrane (CM), causing pore formation that leads to permeabilization of the cell.

Class II is a large and diverse group of unmodified, non-lantibiotic bacteriocins. Compared to the class I bacteriocins, the class II bacteriocins are structurally simpler because they don't undergo PTMs. Because of this, they don't need any enzymes other than a leader peptidase and/or a transporter protein for their maturation (Alvarez-Sieiro et al. 2016). This class consists of five subclasses: Class IIa, IIb, IIc, IId and IIe.

Class IIa consists of the pediocin-like bacteriocins, which are named after the first characterized class IIa bacteriocin: pediocin PA-1 produced by *Pediococcus acidilactici* (Hassan et al. 2012). Bacteriocins in this class have a strong ability to kill listeria, and they are produced by a variety of LAB. They have a conserved, cationic N-terminal domain (YGNGV) and a less conserved, hydrophobic/amphiphilic C-terminal domain linked together with a flexible hinge. Despite having this conserved N-terminal end, the activity and target specificity of the bacteriocins differ within the class (Nes et al. 2007). Class IIa bacteriocins kill target cells by using the mannose phosphotransferase system (Man-PTS) as a target receptor, creating pores in the cell membrane resulting in loss of proton motive force and leakage of intracellular components (**Figure 1-2**) (Cui et al. 2012; Diep et al. 2007).



Source: Diep et al. 2007

Figure 1-2 A model showing the insertion of class a IIa bacteriocin into the cell membrane using the Man-PTS as a receptor (1-2) triggering permeabilization of the cell membrane (3).

Class IIb contains the non-lantibiotic, two-peptide bacteriocins. Bacteriocins in this class have little or no activity unless the two peptides are present at roughly equal amounts. To be classified as class IIb bacteriocins, the genes encoding the peptides should be next to each other in the same operon, followed by a single immunity gene (Nes et al. 2007; Nissen-Meyer et al. 2010). The first isolated and characterized class IIb bacteriocin was lactococcin G, which kills cells by making the cell membrane permeable to monovalent cations like Na^+, Li^+ and K^+, but not for divalent cations (like Mg^{2+}) or anions. This leads to disruption in the electrochemical potential and cell death (Nissen-Meyer et al. 2010).

Class IIc contains the unrelated, non-pediocin-like, single-peptide bacteriocins (Alvarez-Sieiro et al. 2016). One example is the narrow spectrum bacteriocin lactococcin A which is produced by *Lactococcus lactis*. This is one of the first biochemically characterized bacteriocins from *L. lactis* and is shown to be active only against other lactococci (Holo et al. 1991; Nes et al. 2007).

Class IId is the leaderless bacteriocins that have been found in several Gram-positive bacteria, first identified in *Streptococcus* and *Staphylococcus* (Nes et al. 2007). These bacteriocins are produced without an N-terminal leader peptide, thus they don't have the sequence that is used as recognition site for modifications and secretion. These bacteriocins are generally exported by an ABC transporter, but detailed mechanism is not yet fully understood (Nes et al. 2007).

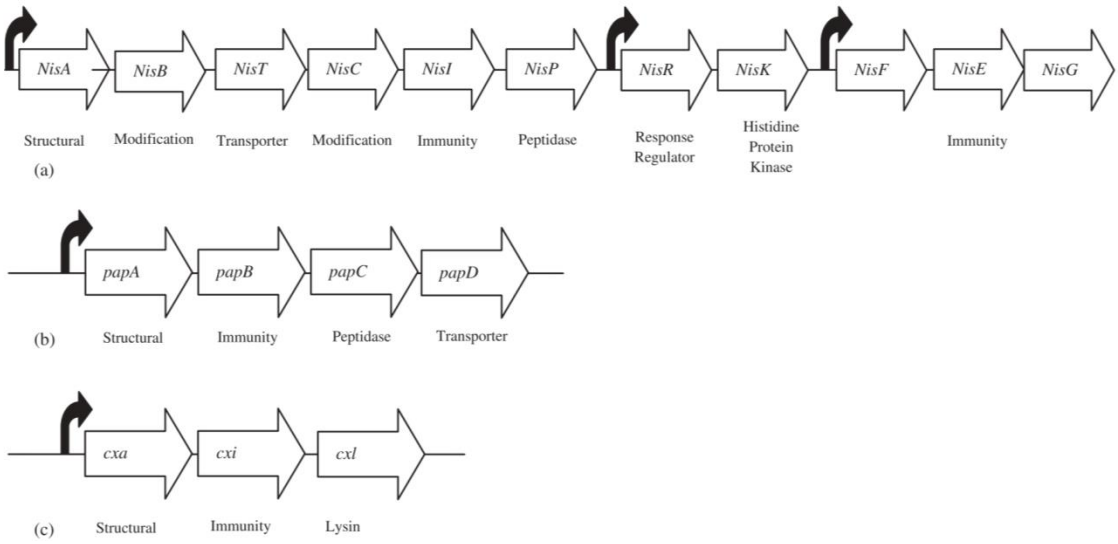
Class IIe is the class with the larger protein-derived bacteriocins. In eukaryotes both histones (Birkemo et al. 2003) and lactoferrin (Gifford et al. 2005) are sources of such antimicrobial peptides. It has also been shown that some propionic acid bacteria produce bacteriocins by

degradation of proteins. The best studied class IIe bacteriocin is propionicin F which is a product of both N-terminal and C-terminal modifications of a precursor protein (Nes et al. 2007).

Class IV contains the circular bacteriocins which are post-translationally modified. However, these bacteriocins differ from the lantibiotics because they require a more complex synthesizing apparatus (Maqueda et al. 2008). In addition, class IV bacteriocins are covalently linked head to tail, making them cyclic of nature. The most studied bacteriocin in this group is enterocin AS-48, produced by *Enterococcus faecalis*, which permeabilizes the cytoplasmic membrane leading to cell death due to loss of the electrochemical gradient of the target cell (Nes et al. 2007).

1.3 Biosynthesis & regulation of bacteriocins from LAB

The process of bacteriocin synthesis and maturation differ between classes, but they all depend on a dedicated locus. These loci can be quite different, but with some common features; they always contain a structural gene encoding the bacteriocin(s), the immunity gene(s), and gene(s) encoding the transporter protein with associated proteins (**Figure 1-3**) (Eijsink et al. 2002; Snyder & Worobo 2014). The immunity gene(s) are normally co-expressed with the bacteriocin genes, making the producer cell immune to its own bacteriocin (Eijsink et al. 2002).



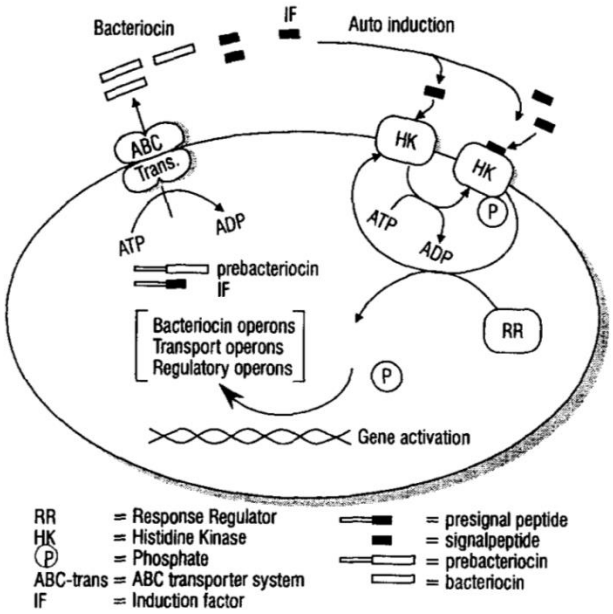
Source: Snyder and Worobo (2014)

Figure 1-3 A representation of the differences in gene clusters for (a) nisin, (b) pediocin and (c) colicin. Common for them are the structural gene of the bacteriocin and the immunity gene encoding the protein that makes the cell immune to its own bacteriocin.

Many class I and class II bacteriocins are secreted from the cell with the same mechanism; by using double glycine-leader peptides that are recognized by a dedicated ABC-transporter and an accessory protein which is specific for peptides containing this leader sequence. In addition, this ABC-transporter cleaves off the leader sequence as the peptide is transported out of the cell (Eijsink et al. 2002).

Production of bacteriocins requires a lot of energy, and it isn't always advantageous for the bacteria to constantly produce bacteriocins. Thus, in some cases, the cells need a system that regulates the production in addition to communicate to the nearby cells when to produce bacteriocins. This communication is made possible by special signaling molecules, often called pheromones, that both synchronize group behavior (quorum sensing), and induce bacteriocin production (Dobson et al. 2012; Nes et al. 1996).

For class I bacteriocins, the bacteriocins are believed to function as signaling molecules themselves, inducing the transcription of bacteriocin genes, while the class II bacteriocins uses a non-bacteriocin molecule as the signaling peptide (**Figure 1-4**) (Eijsink et al. 2002). For example, research has found that the lantibiotic nisin functions both as a bacteriocin and a signaling molecule, inducing its own transcription (Kleerebezem et al. 1997).



Source: Nes et al. (1996)

Figure 1-4 A schematic overview of the regulation and production of class II bacteriocins in a cell. Here, the signal peptide (IF) acts as a pheromone that activates transcription of the bacteriocin-genes in both the producer cell and neighboring cells of the same or closely related strains.

1.4 Practical usage of bacteriocins

Bacteriocins are currently used in food preservation to increase shelf-life, and extensive research is being done on the medical potential of bacteriocins. The first bacteriocin that has been approved for use as a food additive is the class I lantibiotic nisin produced by *L. lactis*, which was used as a preservative in processed cheese products (Delves-Broughton et al. 1996). Nisin was given the European number E234 in 1983 and approved as a food additive due to the fact that it is easily degraded with proteases, presents no risk to humans and it doesn't alter the properties of the food (Gharsallaoui et al. 2016).

Research have also shown that nisin can be used in a wide range of liquids and solid foods like meat-, dairy- and seafood products in order to either prevent the contamination of harmful LAB, inhibit pathogenic Gram-positive bacteria, or prevent contamination by spore forming Gram-positive bacteria such as *Clostridium botulinum* (Gharsallaoui et al. 2016). Nisin can be used either alone or in combination with other factors such as other antimicrobial agents or physical treatments.

There are several types of nisin which has slightly different biological properties. The main types of nisin are A, Z and Q, where nisin A and nisin Z are the most used. These two differ in only one amino acid in position 27, where nisin A has a histidine, and nisin Z has an asparagine. This makes nisin Z more soluble than nisin A at pH levels above 5.0 and thus favorable for use in food preservation (Rollema et al. 1995).

Currently, two bacteriocins are being produced commercially; nisin and pediocin PA-1. Nisin is being marketed under the brand Nisaplin™ and pediocin PA-1 under the name Alta™ 2431. Pediocin PA-1 is used in the preservation of both fresh and fermented meat products, and has shown to be very effective in combination with modified atmospheric packaging (Deegan et al. 2006).

It is much research on the potential applications of bacteriocins in medicine. One of the reasons for this popularity is that the bacteriocins don't seem to induce antibiotic resistance in the same way, or rate, as traditional antibiotics. This is believed to be because considerable changes in the membrane structure have to be done in order to achieve resistance (Peters et al. 2010), making gain of resistance, in some cases, more than just a simple mutation or a gene- or plasmid transfer (Blair et al. 2014). Resistance can however also be obtained through mutations on the bacteriocin receptor of the target cell.

Studies have shown that bacteriocins can be used to prevent both human- and bovine mastitis (inflammation in the mammary gland) (Fernández et al. 2008; Pieterse & Todorov 2010), and commercial products containing nisin are already under development (ImmuCell 2017). In addition to nisin, lacticin 3147 (produced by *L. lactis)* has shown to inhibit several known mastitis pathogens, and it might be a good alternative to traditional antibiotics in the prevention and treatment of bovine mastitis as well (Ryan et al. 1998).

Ways to use bacteriocins as coating on medical devices, such as catheters, in order to prevent unwanted, antimicrobial growth are also being tested (Bahar & Ren 2013; Cotter et al. 2013). The lantibiotics gallidermin (Kellner et al. 1988) and epidermin (Allgaier et al. 1986), produced by *Staphylococcus gallinarum* and *Staphylococcus epidermidis*, respectively, have in clinical trials been shown to be active against *Proprionibacterium acnes*, which is related to acne (Bonelli et al. 2006). Colicins, a family of cytotoxins produced by *E. coli*, have shown to possess cancer inhibiting features, making it a possible tool in future cancer treatments (Lancaster et al. 2007).

Often, antibiotics alone aren't sufficient for treatment of a disease. Several studies have shown that combinations of bacteriocins and traditional antibiotics have a synergetic effect. One study done by Giacometti et al. (2000) showed that nisin in combination with different antibiotics showed bactericidal effects on methicillin-resistant *Staphylococcus aureus* (MRSA). Another study by Joo et al. (2012) showed that nisin might function as a therapeutic for treating head and neck squamous cell carcinoma (HNSCC) by inducing apoptosis, cell cycle arrest, and reducing cell proliferation in HNSCC cells.

Although bacteriocins might appear to be the solution to the emerging antibiotic resistance problem, some issues still have to be sorted out before they can be used in big scale, at least in medicine. The trait that makes bacteriocins useful in food and feed is also one of the traits that makes it difficult to use them in medicine; they are easily degraded by proteolytic enzymes (Zacharof & Lovitt 2012), thus will degrade quickly in the human body. For some diseases, this is advantageous because the bacteriocin only targets where it is needed. However, for other diseases, the bacteriocins will need to work over a longer period of time, making the rapid degradation a problem (Jenssen et al. 2006).

Even though many bacteriocins have been discovered, the need for bacteriocins with a wider inhibition spectra, increased stability and increased specificity is increasing. This, in combination with the increased bacterial resistance against traditional antibiotics, is the main

motivation for the continued research on bacteriocins and their potential use in pharmaceuticals, food and feed.

1.5 Pathogens used for screening

For this study, two different pathogens were chosen for the screening, the Gram-positive *S. aureus* and the Gram-negative *Pseudomonas aeruginosa*. *S. aureus* is an important pathogen because of the combination of its invasiveness and the emergence of antibiotic resistant strains such as the methicillin-resistant *S. aureus* (MRSA) which through horizontal gene transfer has acquired resistance to all known penicillins (Aires de Sousa & Lencastre de 2004; Le Loir et al. 2003). *P. aeruginosa* is an equally important pathogen mainly because of its natural resistance to most traditional antibiotics due to its low outer membrane permeability (being Gram-negative) and an active efflux pump (Stover et al. 2000).

S. aureus is a rod-shaped bacterium that can be both harmless (commensal) and pathogenic for both humans and animals, depending on the environment and strain (Lowy 1998). *S. aureus* commonly colonizes the nose, throat and skin of humans and animals (Williams 1963), and about 30% of the human population is colonized without necessarily being sick (Tong et al. 2015). In humans, *S. aureus* can cause bacteremia (bacterial infection in the blood), osteoarticular (bone and joint) infections, and infections connected to prosthetics, skin and soft tissues, in addition to pneumonia and meningitis (Tong et al. 2015). Skin and soft tissue infections (SSTIs) caused by *S. aureus* can range from harmless, local inflammations to life-threatening, necrotizing fasciitis (flesh-eating disease) (Tong et al. 2015). *S. aureus* is together with *E. coli* and *Streptococcus uberis* also an important factor in bovine mastitis (Bradley 2002).

S. aureus produces several virulence factors such as hemolysins, leukocidins, proteases, enterotoxins and immune-modulatory factors which are regulated during growth (Oogai et al. 2011). Enterotoxins, produced by some strains of *S. aureus*, can cause food poisoning, and it is one of the leading causes of gastroenteritis (inflammation of the stomach and intestines) caused by the consumption of contaminated food (Le Loir et al. 2003).

P. aeruginosa is a Gram-negative, rod-shaped bacteria that can be found in a variety of places, including soil, plants, and animal tissues (Stover et al. 2000). As an opportunistic pathogen, it is commonly found in patients with reduced immune system due to, but not limited to; burn wounds, cystic fibrosis and after organ transplants. *P. aeruginosa* is also connected to urinary tract infections, lower respiratory tract infections and bacteremia (Bodey

et al. 1983). The factors that make *P. aeruginosa* virulent can be divided into two categories, extracellular and cellular. The extracellular factors include proteases, exotoxins and phospholipase, while the cellular factors are the pili, slime polysaccharide and lipid A, amongst others. The exotoxin is believed to be the most toxic component produced by *P. aeruginosa* (Bodey et al. 1983).

1.6 The aim of this study

The main aim of this study was to search for bacteria capable of producing antimicrobial peptides against *S. aureus* and/or *P. aeruginosa* in samples retrieved from fermented fruit and vegetables, and characterize the producers using methods in molecular biology.

An outline of the work done in this study is illustrated in **Figure 1-5**, and consisted of screening, spot-on-lawn inhibition assay, fingerprinting, sequencing, and fermentation profiling. For the bacteria obtained from screening against *P. aeruginosa* only screening and spot-on-lawn assay was done.

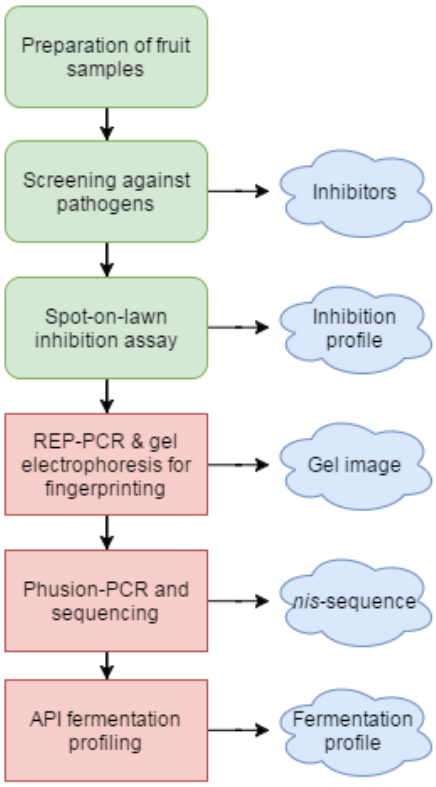


Figure 1-5 Flowchart illustrating the workflow used in this study. The three first steps (green) was done for both *P. aeruginosa* and *S. aureus* while the last three steps (red) was only done with the bacteria found in the screening against *S. aureus*. The blue clouds indicate what kind of results each step yielded.

2 Materials & Methods

2.1 Bacterial growth media

When working with bacteria, special growth media are needed, and in this study, different types of agar, soft agar and broth were used. The growth media were prepared by following the recipe provided by the manufacturer, followed by autoclaving and appropriate storage until needed. Solid growth media contained 1.5 % agar powder, while soft agar contained 0.8% agar powder. Growth media used in this study includes de Man, Rogosa and Sharpe (MRS) (Oxoid) and Brain-heart infusion (BHI) (Oxoid), and the concentrations used were 52 g/L for MRS and 37 g/L for BHI, unless stated otherwise. Every overnight (ON) incubation was done under aerobic conditions unless stated differently.

All work done during this study with high risk of being contaminated was carried out in sterile work benches with fume hood using sterile equipment and disposable gloves.

2.2 Collection of samples

The samples originated from fermented fruit and vegetables which were prepared from 50 different types of fruit and vegetables (**Table A-1**, appendix) bought from a Turkish shop in Hauketo, Oslo. For each type, 20-50 g of chopped fruit was put into a container (bag/cup); two containers per fruit. Tap water was added to one container, while the other container had both water and a tea spoon of salt to create two different environments for each fruit. The containers were then left to ferment/decay for 3 weeks at varying temperature (10-25℃ in an outdoor storage room) before 1 mL of the liquid was squeezed out and mixed with glycerol (to 15-20%) and kept at -80℃ for storage until further use. Glycerol is used because it prevents the formation of ice crystals that would have destroyed the cells in the samples.

2.3 Screening for bacteria with antimicrobial activity

The screening was done in two rounds, one round with *S. aureus* as indicator strain (a combination of two strains B1561 and B1562) (1^{st} screening) and a second round with *P. aeruginosa* PAO1 (B1612) as indicator strain (2^{nd} screening). The protocol for each round was the same (described below), but with the exception that the bacteria from the samples and the indicator *P. aeruginosa* were grown aerobically at 30℃, while the samples and indicator *S. aureus* were grown anaerobically at room temperature. *P. aeruginosa* needs aerobic conditions to grow, while *S. aureus* is a facultative anaerobic bacterium, thus grows well under both aerobic and anaerobic conditions. The last step for *S. aureus* could therefore have been aerobic, but anaerobic conditions were chosen for simplicity since the samples already

were placed in an anaerobic growth chamber in addition to being favorable conditions for LAB.

ON culture of the indicators was made from glycerol stock by using a sterile toothpick to scrape of cells from the frozen stock culture and drop into a culture tube containing 5 mL of BHI growth medium and incubate ON at 30℃. When creating the ON culture of *P. aeruginosa*, it was discovered that in order to achieve proper growth on the plates, the ON culture had to be freshly made from the frozen stock and not carried over from a previous, non-stock culture. It was also important to keep the soft agar at appropriate temperature for *P. aeruginosa* because it is a Gram-negative bacterium with lower resilience to heat than the Gram-positive *S. aureus*.

Before starting the main screening, two different methods were tested in order to find the optimal one. Both methods were selective for LAB, and required three days to complete (**Figure 2-1**). Both methods are described below, but the second method is more thoroughly described as it gave the best results.

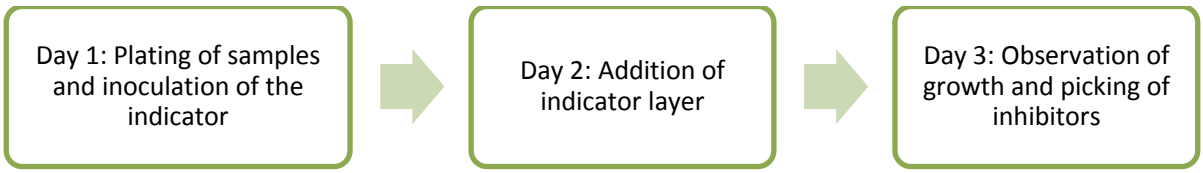


Figure 2-1 Overview of the screening procedure used in this study

The first method consisted of using a sterile inoculating loop to streak some of the fruit or vegetable sample onto a MRS plate using a pattern aiming to dilute the sample with each streaking. After streaking, the plates were incubated anaerobic ON at 30℃ before it was added 5 mL melted BHI soft agar containing the indicator ON cultures (750 µL ON culture in 150 mL BHI soft agar). The plates were allowed to dry and solidify before new ON growth at 30℃. Growth and inhibition was observed the following day.

The second method consisted of using several layers of different media as illustrated in **Figure 2-2**. The samples were prepared as shown in **Figure 2-3** using 0.9% NaCl and sterile culture tubes containing 5 mL melted MRS soft agar. The soft agar was kept molten by keeping the culture tubes containing the agar in a water bath (Julabo) set to 48℃. Between each dilution, the tubes were thoroughly vortexed (Scientific Industries). Each of the three dilutions was poured onto marked 26 g/L MRS agar plates and allowed to solidify before 5 mL MRS soft agar was added as a middle layer. This was to avoid smearing and mixing of the

colonies when the top layer containing the indicator bacteria was to be added the next day. The agar plates were again allowed to solidify and dry before being placed in an anaerobic growth chamber along with AnaeroGen™ bags (Thermo Scientific) to create an anaerobic environment. The plates were incubated at room temperature in order to get better resolution of the inhibition zones due to slower growth of the bacteria, and thus less production of potential antimicrobials. After ON incubation, 5 mL of melted BHI soft agar with the indicator were added to each plate (750 mL ON culture in 150 mL BHI soft agar) using PipetBoy (Integra). Plates were again incubated anaerobically ON at room temperature for the screening against *S. aureus*, and aerobically at 30℃ for the screening against *P. aeruginosa*.

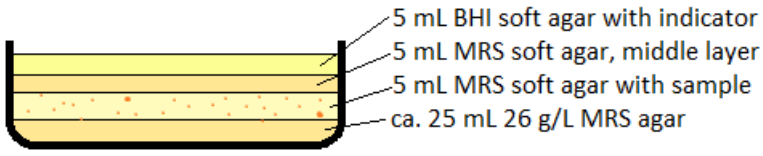


Figure 2-2 Schematic overview of the layers used for the second, preferred screening method. Here, the layer called "middle layer" acts as a barrier between the sample and the indicator to prevent addition of the last layer to interfere with the growing colonies.

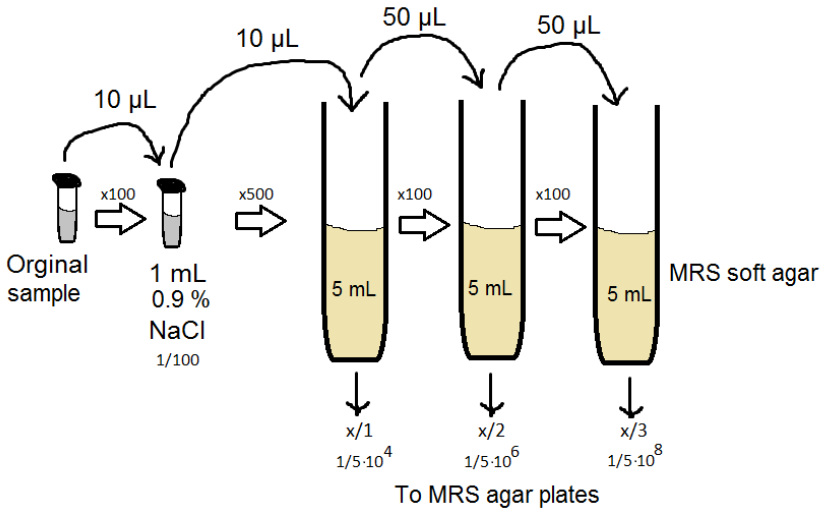


Figure 2-3 A simple diagram illustrating the dilution series used in the second screening method resulting in three different dilutions; x/1, a $5 \cdot 10^4$ dilution; x/2, a $5 \cdot 10^6$ dilution; and x/3, a $5 \cdot 10^8$ dilution. 0.9% NaCl is used for the initial dilution, while 5 mL MRS soft agar is used for the last three to be poured onto MRS agar plates.

After incubation the growth and inhibition zones were observed and photographed. Colonies showing inhibition of the indicator were picked with a sterile toothpick, streaked on a MRS agar plate, and incubated at 30℃ ON in order to get single colonies. A single colony was then picked using a sterile toothpick and dropped into 5 mL MRS medium for ON growth at 30℃ to get a pure culture. To prepare for long-term storage, 0.8 mL of this ON culture was mixed with 0.4 mL 45% glycerol in Cryo-tubes (Sarstedt), to a final amount of 15 % glycerol, and kept a -80℃ until further use.

2.4 Spot-on-lawn inhibition assay

The pure cultures obtained from both of the screening rounds were then tested for purity and inhibition ability by conducting a spot-on-lawn inhibition assay. About 25 µL ON culture of the desired indicator was added to 5 mL melted BHI soft agar and spread evenly on an agar plate (MRS or BHI). One drop (approx. 1 µL) of ON cultures of the bacteria to be tested were added to marked spots on the plate and allowed to dry before ON incubation at 30℃.

The samples from the first screening were tested against the two strains of *S. aureus* used for screening (B1561 & B1562), a garvicin KS producing *Lactococcus garvieae* (B1310) and a nisin Z producing *L. lactis* (B1574) on MRS agar plates, while the samples obtained from the second screening were tested against *P. aeruginosa* (B1612) on both MRS- and BHI plates.

2.5 DNA methods

Isolation of genomic DNA

The samples obtained from the first round of screening were studied further with polymerase chain reaction (PCR) and sequencing. In order to do this, DNA had to be released from the cells by using mechanical lysis with the FastPrep®24 machine (MP Biomedicals).

Cells were collected by centrifugation of 5 mL ON culture at maximum speed (13 000 rpm) for 3 min. The supernatant was discarded and the cell pellet was washed with 300 µL TBS-buffer with pH 7.4 before a new centrifugation for 3 more min at maximum speed. Again, the supernatant was discarded and the cell pellet was resuspended in 250 µL cold buffer P1 (Qiagen). The resuspension was then transferred to a FastPrep-tube containing approx. 0.5 g acid washed glass beads (<106 nm, Sigma). The tube was secured in the FastPrep®24 homogenizer, and run 3 times for 20 sec at speed 4 m/s with 1 min break between runs. This was in order to limit the heating of the samples caused by friction. After homogenization, the tube was spun down, and the supernatant (now containing free DNA) was transferred to a new 1.5 mL eppendorf tube.

The extracted DNA then had to be purified, and this was done by using Miniprep columns (Qiagen) in three main steps; binding of DNA to the column, ethanol wash, and eluation with sterile, filtrated water.

It was added 250 µL Solution II to the eppendorf tube containing the free DNA, and the tube was inverted 4-6 times to mix the solution. Then, 350 µL Solution III was added and the tube was again inverted a few times. The eppendorf tube was centrifuged at 13 000 rpm for 10 min and the liquid was transferred to a Miniprep column placed in a collection tube. This column and tube was centrifuged at maximum speed (>10 000 rpm). The flow-through was discarded before 500 µL isopropanol was added in order to bind the DNA even tighter to the column. The tube was again centrifuged at maximum speed for 1 min and the flow through was thrown away. To wash out the remaining proteins in the samples 750 µL ethanol was added to the column and the tube was centrifuged for 2 min at maximum speed. The flow through was again discarded and the column was allowed to dry with the lid off in order to remove excess ethanol. The column was then transferred to a new eppendorf tube and 30 µL of sterile, filtrated water was carefully added to the middle of the column. After waiting 1 min, the column and eppendorf tube was centrifuged for 1 min at maximum speed to eluate the DNA. After centrifugation, the water in the eppendorf tube contained the purified DNA.

The quality and concentration of DNA was measured by using NanoDrop (as described below), and the purified DNA samples were stored at -20℃ until further use.

NanoDrop

To quantify the amount and purity of isolated DNA and PCR-products, the NanoDrop ND-1000 (NanoDrop Technologies) was used with the elution buffer as a blank sample. To measure a DNA concentration, 2 µL sample was added to the sensor and absorbance at λ = 280 nm was measured. It is at this wavelength the nucleotides have the highest absorbance. The purity was given as a 260/280 absorbance ratio, and should be between 1.8 and 2.0 in order to not have too much proteins or RNA in the sample. Higher values indicate RNA contamination in the sample, while too low values indicate protein contamination.

The results from NanoDrop were used to normalize the DNA concentrations. For the rep-PCR reaction, a DNA concentration of 100 ng/µL was used, while for the PCR with the Taq polymerase a concentration of 50 ng/µL was made.

2.6 Polymerase chain reaction (PCR)

Two different variations of PCR were used in this study; repetitive element palindromic-PCR (rep-PCR) using OneTaq DNA polymerase (New England Biolabs), and a regular PCR amplification using Phusion® DNA polymerase (New England Biolabs).

Rep-PCR is a method used to generate DNA fingerprints that can be used to separate bacterial species and strains by using a specific set of primers (REP-1R and REP-2I) that match the repetitive extragenic palindromic (REP) elements in the genome (Woods et al. 1993).The primers contain the nucleotide inosine (I) which can form Watson-Crick base pairs with any of the four natural bases (adenine (A), tyrosine (T), cytosine (C) or guanine (G)), however favoring the base pairs I:C, I:T and I:A which has the greatest stability (Watkins & SantaLucia 2005). This property makes inosine ideal to use in primers that needs to bind to a variety of related sequences such as the REP-elements.

For the amplification of the *nis*-gene using regular PCR, Phusion DNA polymerase was used instead of Taq polymerase in order to achieve lower error rate because the amplicon was to be sequenced (New England BioLabs 2017). Specific primers flanking the *nis* gene (nisZ-R and nisZ-F) were designed using BLAST to identify the regions up- and downstream of the *nis* gene (sequences shown in **Table A-2** in the appendix).

Rep-PCR

A PCR reaction mix containing OneTaq buffer (New England Biolabs), nucleotides, primers and Taq DNA polymerase was made according to **Table 2-1** below. For each well, 20 µL of the master mix and 5 µL of template DNA (concentration 100 ng/µL) was added. The content of each well was mixed, the strips were placed in a PCR machine (Bio-Labs), and the program described in **Table 2-2** was used to amplify segments of the genome.

Table 2-1 Composition of the PCR master mix used in the rep-PCR. Primer sequences can be found in the appendix (**Table A-2**).

Solution	Volume per 2 PCR tube á 25 µL
OneTaq standard buffer	10 µL
10 nM dNTP	1 µL
10 nM REP-1R primer	5 µL
10 nM REP-2I primer	5 µL
100 ng/µL DNA template	5 µL
Taq DNA polymerase	0.5 µL
dH_2O	18,5 µL
Total	50 µL

Table 2-2 The program used for the rep-PCR fingerprinting of the isolated DNA

Temperature	Duration	Cycles	Action
95 ℃	7 min	1	Initial denaturation
94 ℃	1 min		Denaturation
41 ℃	1 min	35	Primer annealing
65 ℃	3 min		Primer extension
65 ℃	16 min	1	Final extension
4 ℃	Hold	-	Storage

Phusion-PCR

It was made a PCR reaction mix containing buffer, dNTPs, primers and Phusion-polymerase according to **Table 2-3** below, 45 µL per reaction. Template DNA (concentration 50 ng/µL) was added to each well, and the PCR program described in **Table 2-4** below was used to amplify the *nis*-gene.

Table 2-3 Composition of the PCR master mix used for the Phusion-PCR

Solution	Volume for total 50 µL
Phusion buffer	10 µL
10 nM dNTP	1 µL
10 nM nisZ-F primer	2.5 µL
10 nM nisZ-R primer	2.5 µL
50 ng/µL template DNA	5 µL
PhusionDNA polymerase	0.5 µL
dH_2O	28.5 µL
Total	50 µL

Table 2-4 The PCR program used to amplify the *nis*-gene using Phusion DNA polymerase.

Temperature	Duration	Cycles	Action
95 ℃	7 min	1	Initial denaturation
94 ℃	1 min		Denaturation
55 ℃	1 min	30	Primer annealing
65 ℃	3 min		Primer extension
65 ℃	16 min	1	Final extension
4 ℃	Hold	-	Storage

2.7 Agarose gel electrophoresis

To visualize the products after PCR, agarose gel electrophoresis was used. For the products from the rep-PCR, the goal was to identify unique profiles, while for the Phusion-PCR products the aim was to check if there was a band corresponding to the *nis*-gene.

It was tested with different amounts of agarose before settling with 1.8 % as the best composition for the rep-PCR products and 1% for the Phusion-PCR products. This gave the best separation of the bands. It was added 3 µL peqGreen dye to 50 µL gel in order to make it possible to visualize the DNA using UV-light.

The gel was made using the appropriate equipment and placed in a gel electrophoresis chamber together with 1xTAE buffer. Samples were mixed with loading buffer and loaded to the gel with 5 µL ladder on each side (1 kb ladder for fingerprinting and 100 bp ladder for *nis*-gene). For the rep-PCR products, 12.5 µL gDNA was mixed with 1 µL loading buffer, while for the Phusion-PCR products, only 4 µL sample was mixed with 1 µL loading buffer. This was because the DNA (only amplified *nis*-gene) is more concentrated than the rep-PCR products, needing less sample material to get a strong band.

The agarose gel electrophoresis was run at 80 V and 80 mA for about 180 min for the rep-PCR products, and approx. 30 min for the Phusion-PCR products. The bands were visualized and photographed using UV light (Bio-Rad).

2.8 Purification and sequencing of the *nis* gene

Based on the results from the rep-PCR, a selection of the samples representing each group was chosen for sequencing. The samples were first run on a gel to check that the PCR reaction had amplified the *nis* segment of the DNA, before the samples were purified using DNA-binding paramagnetic AMPure XP beads (1:1 ratio) (Beckman Coulter).

The product from the PCR reaction was added to a new eppendorf tube together with 50 µL of the AMPure beads and mixed well in order to bind DNA to the beads. After being incubated for 10 min at room temperature, the tube was placed in a magnetic stand for 2, min making the paramagnetic beads gathering on one side. The supernatant was removed and the beads were then washed with 200 µL 70% ethanol to remove leftover contaminations after the PCR reaction (primers, dNTPs, buffer and proteins). After washing, the beads were allowed to dry before DNA elution. This was done by adding 25 µL of sterile, filtrated water with the tube not in the magnetic stand. Now, the water bound to the beads because of higher affinity to the beads than the DNA, thus making DNA being released to the supernatant. By placing

the tubes in the magnetic stand and waiting for a few minutes, the supernatant contained the free, purified DNA which was transferred to a new eppendorf tube.

After purification the DNA concentration was measured using NanoDrop, and this information was used to prepare the samples to be sent for Sanger sequencing by the company GATC Biotech. The samples were prepared by combining 10 µL of DNA (concentration 20-80 ng/µL) and 5 µL nisZ-R primer (concentration 5 µM) to a total volume of 15 µL.

2.9 Pulsed field gel electrophoresis (PFGE)

A selection of the isolated bacteria was sent to a collaborating lab in Serbia for pulsed field gel electrophoresis (PFGE). The purpose of this was to more accurately fingerprint the samples to see if there were any differences in the genome between the different bacteria not being detected by the rep-PCR fingerprinting.

The principle of PFGE is similar to that of regular gel electrophoresis, only that the electric field is alternating in more than one direction, hence the "pulsed field"-name. This allows for separation of larger DNA fragments, making it possible to separate DNA fragments as large as 10 megabases (Mb). The advantage of this method over regular gel electrophoresis is that every step is carried out directly on the agarose gel, from lysis of the cells to purification and digestion of the sample. The bands are visualized using UV light, similar to regular gel electrophoresis (Kaufmann 1998).

2.10 Fermentation profile of selected samples

The kit API®50 CHL (Biomérieux) was used to study the fermentation profile for a selection of the isolates from the first round of screening. This kit included a set of strips with cupules containing different carbohydrates and a special medium (50 CHL). This medium contained a pH indicator which showed what type of carbohydrate each bacterium was able to ferment because of the lactic acid produced during fermentation. In total, 13 samples were used in addition to three selected samples from the LMG library as a reference: P3, garvicin KS producer *L. lactis* from milk; D4, nisin A producer *L. lactis*; and garvicin ML producer *L. garvieae* from duck intestine.

Cells were harvested by centrifuging 1 mL ON culture for 5 min at 13 000 rpm. The supernatant was removed and the surface of the cell pellet was washed with 50 µL 50CHL medium (Biomérieux) and spun down for 1 min. The cell pellet was then resuspended in 2 mL 50CHL medium, and 300 µL of this resuspension was transferred to 6 mL 50 CHL medium.

The API kit was assembled as described by the manufacturer by adding 10 mL of dH_2O to the honeycomb pattern in the bottom of the box to create a moist environment. Approx. 100 µL of bacterial suspension were filled in each cupule and the opening was sealed by adding mineral oil (Sigma) to create anaerobic conditions. The lid was placed on the box, and the fermentation chambers were incubated at 30 ℃. Color change was observed at specific time points (24h, 48h and 72h+).

3 Results

3.1 1st screening: *Staphylococcus aureus* as indicator strain

Samples from 50 different fermented fruit and vegetables were used to screen for bacteria with production of antimicrobial components against two different strains of *S. aureus.* Two different methods were tested; streaking and dilution. The dilution method showed better separation of the colonies (**Figure 3-1**), and was thus chosen as screening method for the rest of the study. The specific dilution used resulted in colony numbers varying from 10 to above 700 for the $5 \cdot 10^6$ dilution (x/2), and 0-50 for the $5 \cdot 10^8$dilution (x/3) (**Figure 3-2**).

The majority of the fruit samples didn't have any bacteria showing inhibition of *S. aureus*. Plates having colonies showing inhibition (like those shown in **Figure 3-3**) originated from avocado, sugar peas and dragon fruit, amongst others (**Table A-3**, appendix). In total, 44 colonies (some replicates) were chosen for further studies.

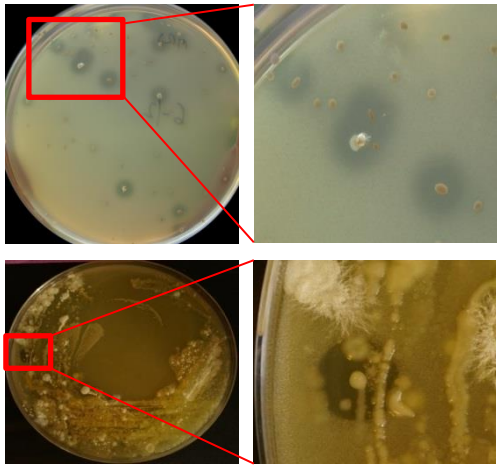


Figure 3-1 Comparison of the streaking method (**bottom**) and the dilution method (**top**). The dilution method was chosen because it showed the best separation of the colonies. The image from the streaking method originates from a plate grown aerobic, thus having growth of aerobic bacteria and fungi as well.

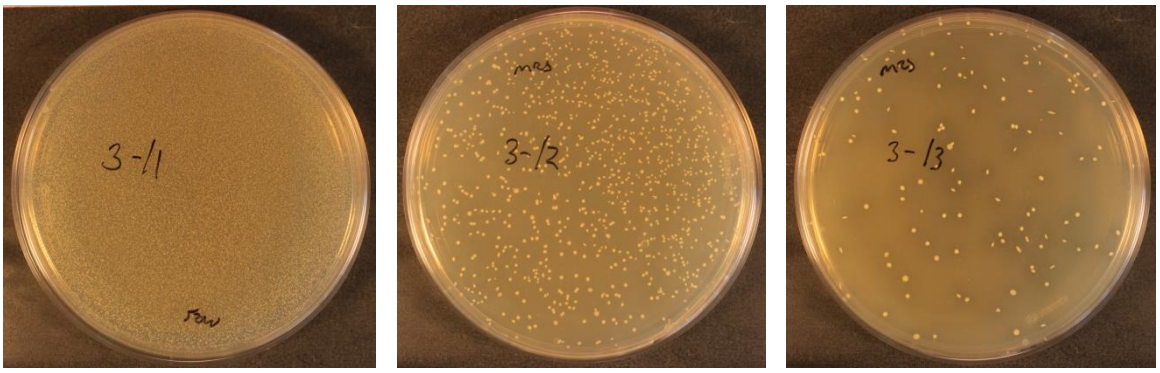


Figure 3-2 Results from the dilution of the orange watermelon sample (3-) against *S. aureus*. The plates show a clear reduction of cells as the sample gets more diluted (from left to right).

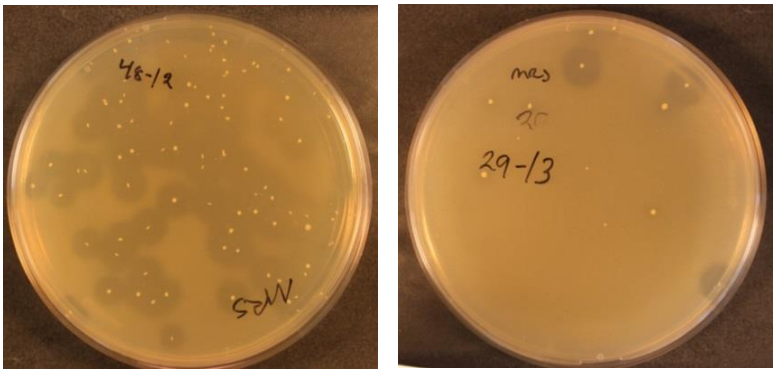


Figure 3-3 Screening assay for two selected samples against *S. aureus*. The screening results from dragon fruit (48-) (**left**) shows that every colony inhibited the two strains of *S. aureus*, while the results from sugar peas(29-) (**right**) shows a more common inhibition pattern, where only some of the colonies inhibited growth of the indicator.

3.1.1 *Spot-on-lawn inhibition assay*

All pure cultures from the first round of screening were tested against other bacteria using spot-on-lawn inhibition assay to check for purity and activity. Here, the isolates were tested against the same two strains of *S. aureus* from screening, garvicin KS producer *L. garvieae* and nisin Z producer *L. lactis*. All of the tested samples showed strong inhibition of both strains of *S. aureus* (**Figure 3-4, right**), and weak inhibition of the garvicin KS producer (**Figure 3-4**, **left**). The isolates had no inhibitional effect on the nisin Z producer (**Figure 3-4**, **middle**), suggesting that the isolates might produce a nisin-like component.

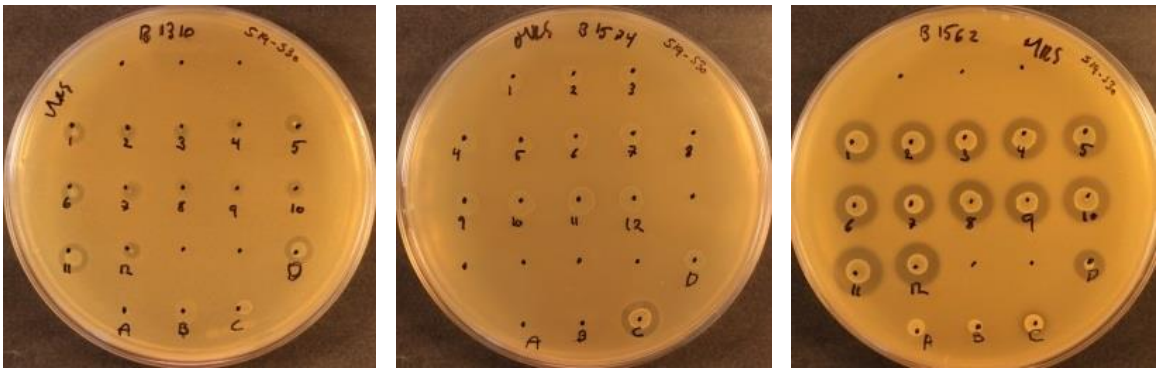


Figure 3-4 Spot-on-lawn assay from a selection of the samples (S19-S30) against *L. garvieae* (**left**), *L. lactis* (**middle**) and *S. aureus* (**right**). The spots A-D corresponds to controls, where A and B are the two strains of *S. aureus*, C is *L. garvieae* (garvicin KS producer) and D is *L. lactis* (nisin Z producer).

3.1.2 *Rep-PCR*

All of the bacteria isolated from the fruit samples were fingerprinted using rep-PCR to see if there was a genetic difference between the isolates. A profile for each isolate was visualized by running the PCR product on a gel. This showed that many of the 44 samples had the same profile, and in total 16 unique band patterns (profiles) were identified. A selection of these can be seen in **Figure 3-5** below. These profiles formed the basis for the selection of samples to be sent for PFGE. The isolates having the same band pattern tended to originate from the same fruit source; for example, isolates S20, S21, S26 and S29 all had the same pattern and originated from dragon fruit.

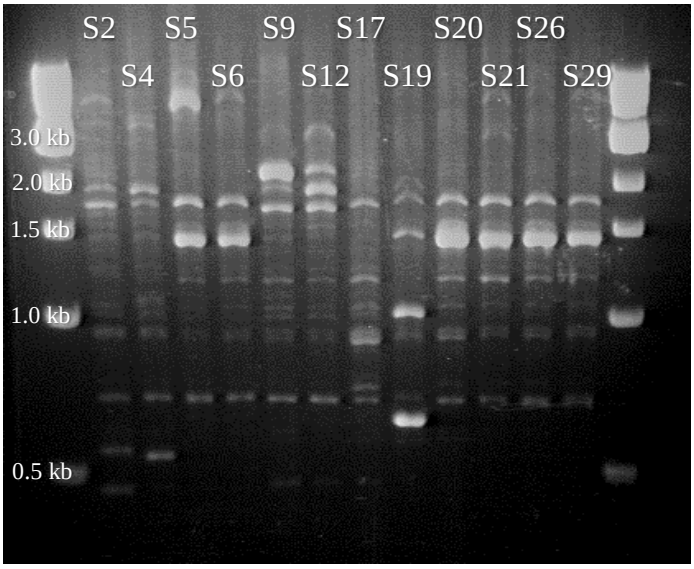


Figure 3-5 Rep-PCR results for a selection of the isolates representing different fingerprints with a 1 kb ladder on each side. Samples showing the same band pattern tended to originate from the same fruit sample; for example the isolates S20, S21, S26 and S29 originated from dragon fruit (48).

3.1.3 *Phusion-PCR & Sanger sequencing*

Based on the screening procedure being selective for LAB, combined with the results from the spot-on-lawn inhibition assay, it was believed that the antimicrobial components produced were some variant of nisin. This was tested by using nisin-specific primers for PCR to amplify the *nis*-gene and sequence the amplicon. The results showed that every single isolate had a strong band with a size corresponding to that of the *nis*-gene (**Figure 3-6**).

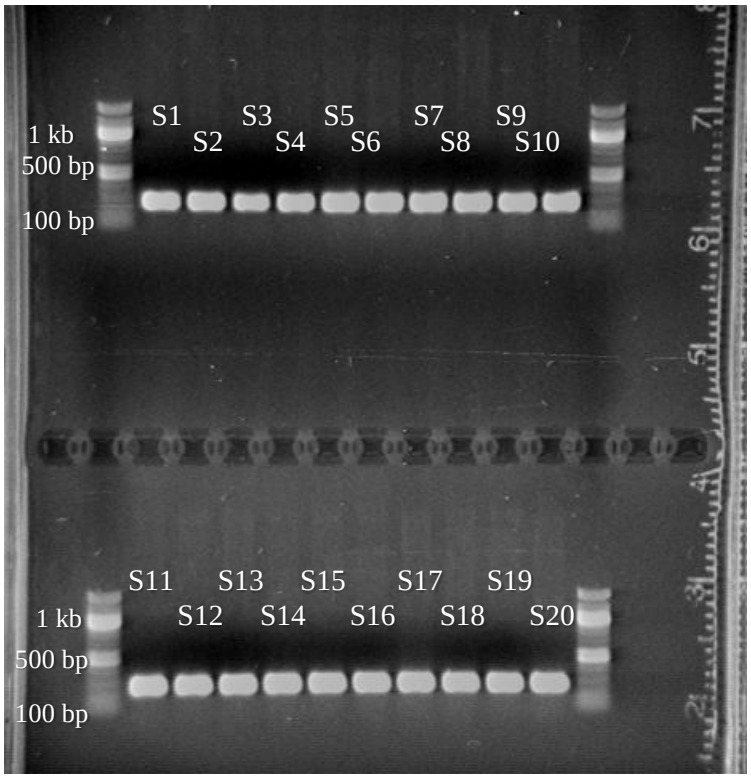


Figure 3-6 Phusion-PCR bands for the isolates S1-S9 (**top**) and S11-S20 (**bottom**) with a 100 bp ladder on each side. The gel shows one clear band corresponding to the *nis* gene for every sample. The length of the fragment (about 200 bp) also corresponds to that of *nis*.

The sequencing results showed that all samples had identical sequence to the known *nisZ*, apart from isolate S4 (from purple aubergine) which had a (unimportant) mutation in the propeptide region; a region which is cleaved of during PTM, thus having no function in the mature peptide (UniProt 2015). The sequences obtained are shown in **Figure A-1** in the appendix, where sequences for both *nisZ* and *nisA* have been retrieved from the NCBI database to act as references.

3.1.4 *Pulsed field gel electrophoresis (PFGE)*

In collaboration with a group in Serbia, a selection of the samples representing different rep-PCR profiles were sent for pulsed field gel electrophoresis (PFGE). The result was a gel image showing a profile for each sample (**Figure 3-7**), and in total there were identified 15 different profiles, some consisting of two or three isolates; [S1+D10], [S4], [S5], [D5], [S7+S8+S9], [S16+S18], [S19], [S20+S26], [D1], [D14+D15], [D16], [D24+D25], [D4], and [NP45]. This grouping matched the grouping found with rep-PCR; isolates from the same fruit source have the same band pattern.

The group in Serbia also ran an activity assay (data not shown) similar to the spot-on-lawn inhibition assay described previously, and sample S5 (from purple aubergine) showed little inhibition of the indicator strain, thus the PFGE result for D5 might be incorrect.

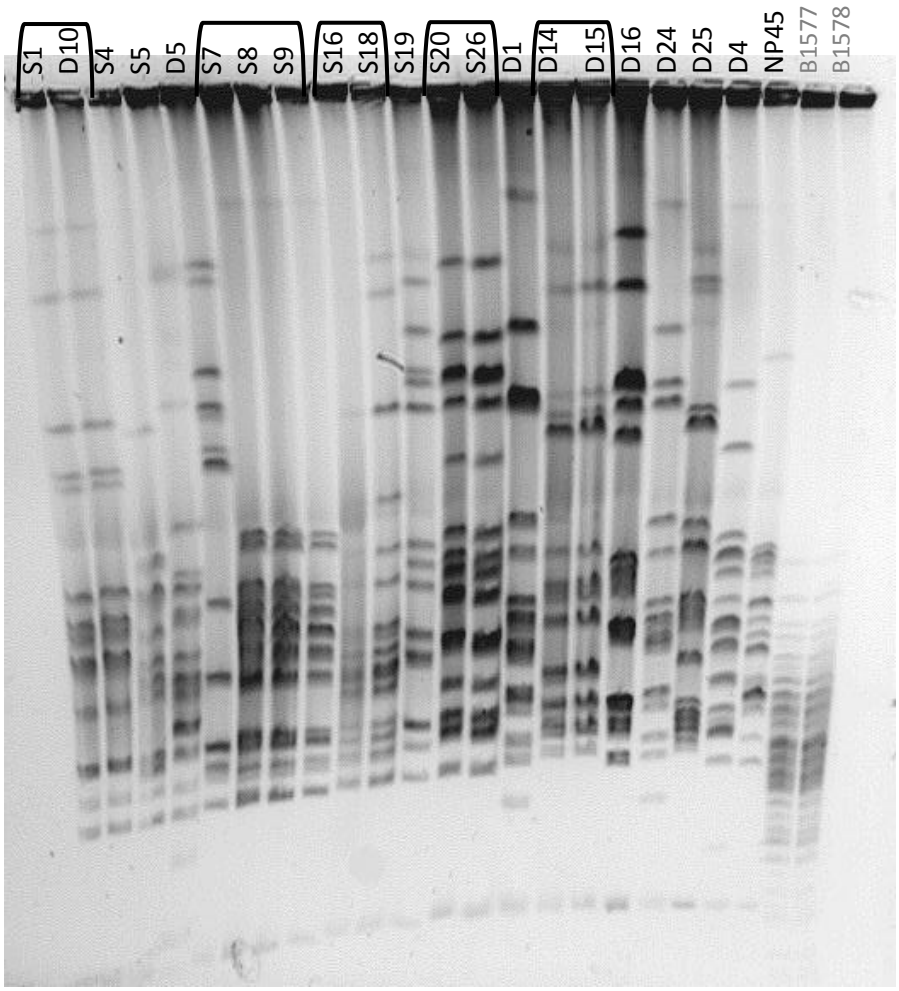


Figure 3-7 Pulsed field gel electrophoresis (PFGE) for bacteria selected based on the rep-PCR results. There are 15 different profiles including the nisin Z producer NP45 from the group in Serbia (excluding the group consisting of B1577 and B1578, both *Pectobacterium*, not of interest in this study).

3.1.5 *Fermentation profile using API®50*

Based on the PFGE results, 13 isolates representing different profiles from the first screening were chosen for API fermentation profiling along with a selection of bacteria from the LMG library. This was done to compare not only the bacteria obtained from the screening with each other, but also to compare them with lactococcal strains originating from different sources (milk (P3) and duck intestine (P4)). A summary of the results are shown in **Table 3-1** (full results in **Table A-5** in the appendix).

The results showed that all of the isolates from the fruit samples were able to ferment D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-saccharose (sucrose), D-trehalose and gentiobiose, although with some variation in efficiency for the different carbohydrates. Some strains showed positive reaction after only 6 hours, while other needed over 48 hours to show a positive fermentation. For example, the samples S19, D4, D5 and D16 needed longer time to achieve positive fermentation of gentiobiose than the other isolates. Some of the bacteria also needed longer time in order to ferment D-lyxose (S4, S18), while others didn't show fermentation of D-lyxose at all (S1, S16).

The main fermentation differences between the bacteria isolated from greens was that only some of the isolates (S18, D1, D14 and D24) showed fermentation of D-raffinose and D-melibiose, in addition to that only D1 and D24 showed (weak) fermentation of L-rhamnose. All but S1, S16 and D24 had slow fermentation of D-lyxose.

Samples D4 (nisin A producer), P3 (garvicin KS producer) and P4 (garvicin ML producer) had a different profile than the rest of the samples, with P4 being the most different from the rest. P4 was unable to ferment several carbohydrates that the other isolates were able to ferment; L-arabinose, D-xylose, D-mannitol, D-lactose and D-saccharose. P3 was more similar to P4 than to the other samples, differing from P4 by being able to ferment D-mannitol, D-lactose and D-saccharose.

None of the samples showed fermentation of glycerol, erythritiol, D-arabinose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, L-sorbose, dulcitol, inositol, D-sorbitol, methyl-αD-glucopyranoside, inulin, D-melezitose, glycogen, xylitol, D-turanose, both D- and L-fucose, D- and L-arabitol, potassium 2-ketogluconate and potassium-5-ketogluconate.

Table 3-1 Selected results from API fermentation profiling of selected bacteria. The full data can be found in the appendix (**Table A-5**). Similar profiles have been placed next to each other.

Carbohydrate	Sample ID															
	S1	S4	S16	S7	S19	S20	D5	D16	D14	S18	D1	D24	D25	P3	D4	P4
L-arabinose	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-
D-xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	-	∓	-
L-rhamnose	-	-	-	-	-	-	-	-	-	-	∓	∓	-	-	-	-
D-galactose	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+	∓
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-mannitol	+	+	+	+	+	+	±	+	+	+	+	+	+	+	-	-
Methyl-αD-mannopyranoside	-	-	-	s+	-	s+	s+	s+	s+	-	-	-	-	-	-	-
D-lactose (bovine origin)	+	∓	±	±	±	±	±	±	±	±	+	+	∓	+	-	-
D-melibiose	-	-	-	-	-	-	-	-	±	∓	±	±	-	-	-	-
D-saccharose (sucrose)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
D-raffinose	-	s+	-	-	-	-	-	-	+	∓	±	∓	-	-	-	-
Amidon (starch)	±	∓	∓	∓	∓	±	∓	∓	±	∓	±	∓	∓	-	±	∓
Gentiobiose	+	+	+	+	∓	+	∓	±	+	+	+	+	+	+	+	+
D-lyxose	-	s+	-	∓	∓	∓	∓	∓	∓	s+	s+	-	∓	-	-	-
Potassium gluconate*	∓	∓	∓	∓	∓	∓	∓	∓	∓	∓	s+	∓	∓	∓	s+	∓

* most of the samples had tiny air bubbles in the well after 24h, and weak reaction

\+ positive reaction after 48h, - no reaction after 48h, ± some reaction after 48h, ∓ some (weak) reaction after 48h

s+ (some) reaction after 72h or later, not present at 48h

3.2 2nd screening: *Pseudomonas aeruginosa* as indicator strain

In addition to the first screening, it was decided to do a second screening with the same fruit and vegetable library against *P. aeruginosa* using the same method as for the first screening. After some trouble getting the indicator to grow as desired, a total of 32 colonies were picked and prepared for storage at -80℃ (**Table A-4**, appendix). The inhibition found during this screening consisted mostly of zones emerging from a collection of colonies instead of individual colonies as seen in **Figure 3-8**.

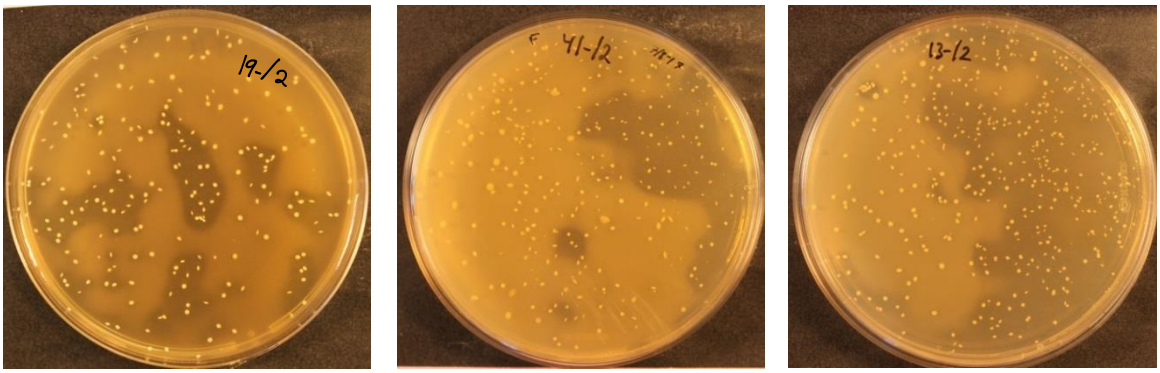


Figure 3-8 Second screening using *P. aeruginosa* as indicator strain. The inhibition zones differ from the ones obtained from the first round of screening, seeming to be a result of several colonies working together. Sample 19 is from plum, 41 from aubergine and 13 from mango.

3.2.1 *Spot-on-lawn inhibition assay*

The pure cultures made from the colonies showing inhibition of *P. aeruginosa* were tested for purity by using the same spot-on-lawn inhibition assay as before, only now, *P. aeruginosa* worked as the "lawn". The assay was run using two different types of agar plates, BHI and MRS. The results for some of the isolates are shown in **Figure 3-9** below, illustrating that all of the pure cultures showed inhibition of *P. aeruginosa*, but with varying degree, on MRS agar plates. None inhibited *P. aeruginosa* on BHI agar plates.

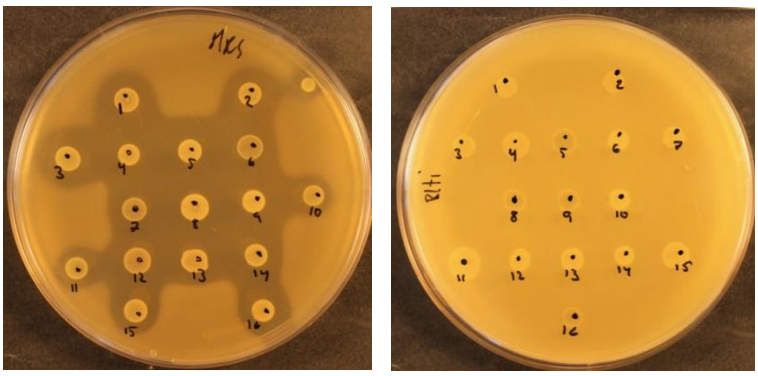


Figure 3-9 Spot-on-lawn assay against *P. aeruginosa* on MRS agar (**left**) and BHI agar (**right**) using some of the isolates (S39-S54).

4 Discussion

In this study, bacteriocin producing bacteria were obtained through a screening process using two known pathogens, *S. aureus* and *P. aeruginosa*. Two different screening techniques were tested, and a dilution-based approach showed to be the optimal method. Although, using a dilution protocol is more time-consuming than streaking, in addition to requiring more plates and growth media, this method was chosen because it gave much better separation of the colonies compared to the streaking method (**Figure 3-1**). Additionally, this method also made it possible to assess a larger number of isolated bacterial cells.

The specific dilutions used in this study were found by trial-and-error with a selection of the fruit and vegetable samples. Optimal dilution will however vary between sample libraries due to samples having different number of cells. The use of a preliminary screening or a dilution test is therefore needed before big scale screening. The dilutions used in this study could possibly have been optimized further to include only two plates, but the varying number of cells in each sample made it the best option to use three different dilutions. With this method, almost every sample had sufficient number of colonies in at least one plate, often the $5 \cdot 10^6$ or $5 \cdot 10^8$ dilution (10 - >700 colonies and 0 - 50 colonies, respectively).

For screening against *S. aureus*, most of the fruit and vegetables having bacteriocin producing bacteria can be characterized as exotic; avocado, aubergine, kelek and dragon fruit. This is also the case for the *P. aeruginosa* screening; rambutan, eddoes, aubergine and jackfruit. Some of the fruit or vegetable samples had bacteriocin producing bacteria that inhibited both of the indicators; dragon fruit, kelek and avocado. It is possible that this is the same bacteria; however, since *P. aeruginosa* is a Gram-negative bacterium the nisin Z producers found during the first screening will normally have little or no effect (Nes et al. 2007). This indicates that the inhibition is either caused by a broad-spectrum bacteriocin capable of inhibiting Gram-negative bacteria, a Gram-negative bacteriocin or simply a different factor such as low pH. Further analyses like fingerprinting and sequencing are needed to determine this.

Screening also showed that the addition of salt affected the microbial composition in the sample; only a few samples had similar results for both parallels (dragon fruit against *S. aureus* and dragon fruit, jackfruit and eddoes against *P. aeruginosa*). These results indicate that the different environment created by adding salt during fermentation has an impact on the microbial community in the fermented samples. However, the observed variation could also be a result of local variation on the surface or inside the fruit or vegetable. It could also be

because the samples were prepared in a normal, non-sterile kitchen, which might be a source to cross-sample contamination.

From the PFGE results (**Figure 3-7**), it can be seen that both of the isolates obtained from dragon fruit against *S. aureus* have the same profile, showing that this strain grows equally well with and without added salt. The isolates obtained from the second screening haven't been characterized, therefor it is not known whether they are the same strain or not until further analyses have been done.

The results from the second screening showed an interesting pattern; the inhibition of *P. aeruginosa* seemed to be a result of collaboration between the different colonies (**Figure 3-8**). This can be a result of quorum sensing, where the bacteriocins, or other signaling molecules, signals to nearby colonies to produce bacteriocins (Nes et al. 1996). However, this only works if the nearby colonies are the same or closely related strains, leading to the assumption that all colonies seemingly producing bacteriocins are the same or closely related strains in these samples.

Spot-on-lawn inhibition assays were done in order to check that the isolates were pure and that they still had antimicrobial activity against the indicator. The bacteria obtained from the second screening were only able to inhibit the *P. aeruginosa* on MRS plates, and not plates containing BHI medium (**Figure 3-9**). This can be explained by the fact that bacteriocin production is controlled by a quorum sensing system (Hoover et al. 2015) that can be inhibited or induced by components in the media (Renye et al. 2016) which will lead to altered bacteriocin production levels.

PCR and sequencing showed that all of the bacteria from the first screening were producers of the lantibiotic nisin, more specifically nisin Z (**Figure A-1**). This corresponds with the results from the spot-on-lawn assay where the growth of the isolated bacteria wasn’t affected by nisin Z (**Figure 3-4**). This is caused by the fact that bacteria are immune to their own bacteriocins (Eijsink et al. 2002), making the nisin producers immune to nisin produced by other bacteria as well. The isolates showed only some inhibition of the garvicin KS producer, showing that nisin Z differs from garvicin KS in mechanism-of-action.

Both of the fingerprinting techniques (rep-PCR and PFGE) showed that isolates obtained from the same fruit samples tended to have the same band pattern, indicating that the bacteria found in the same source are the same strain. The reason for this dominance can be explained

by the fact that this strain inhibits and kills other strains using bacteriocins during fermentation/degradation and/or screening (Reeves 1965).

After profiling and sequencing, bacteria representing the genetically different groups (one from each group) were selected for API fermentation profiling. The results showed that all of the tested bacteria were able to ferment carbohydrates commonly found in fruit and vegetables. Among these are the pentoses; D-xylose, D-ribose and L-arabinose, the hexoses; D-glucose, D-galactose, D-mannose and D-fructose, in addition to sucrose/saccharose (Lee et al. 1970). None of the bacteria were able to ferment D-arabinose, while all but D24 (from white aubergine) were able to ferment L-arabinose. This can be explained by the fact that the L-isomer is more common in nature, making it a better choice as an energy source than the D-isomer (Lee et al. 1970).

The observed variation in ability and efficiency to ferment different carbohydrates might be an effect of the source of the specific bacterium. The carbohydrate composition in the different fruit and vegetables may vary (Nahar et al. 1993; Rahman et al. 1991), in addition to being dependent on the ripeness of the fruit or vegetable (Azizur Rahman et al. 1999).The isolate P4, *L. garvieae*, originating from duck intestine showed a similar fermentation profile to the bacteria with fruit origin. This can partly be explained by the fact that a duck's diet consists of much plant material (Swanson et al. 1985), making the available carbohydrates in the intestine similar to that found in fruit and vegetables (galactose, glucose, fructose and mannose) (Lee et al. 1970).

P4 was not able to ferment lactose, which can be explained by the fact that ducks, and birds in general, are lactose intolerant (Pollock 2002), thus lactose isn't a natural part of the diet. This way, lactose isn't available in the intestine for bacteria to ferment. In contrast, P3, *L. lactis* isolated from milk, showed one of the fastest fermentations of lactose, needing less than 6 hours for a positive reaction. The natural explanation is that lactose is the main carbohydrate found in milk, making it an excellent source for energy. All but P3 showed weak fermentation of amidon (starch), which also can be explained by the fact that milk doesn't contain any starch, making it useless as an energy source for P3.

Conclusion & future

In this study, an efficient screening method that finds bacteriocin producing bacteria in food samples has been established. However, the method seems to be selective for nisin producers when carried out under anaerobic conditions on MRS growth medium. By changing the growth media, temperature and/or anaerobic/aerobic conditions one can alter the selectivity of the screening, causing the result to differ from the nisin producers. Changing the conditions too much will however no longer make the screening specific for LAB.

Additionally, the phenotypic and genotypic characterization of the isolated bacteria was done using rep-PCR, PFGE and fermentation profiling. The results showed that most of the isolated bacteria from screening against *S. aureus* were producers of nisin Z, even though the producers were genetically different.

The next steps related to this work would be to use the same characterizing methods (PCR, PFGE and fermentation profiling) on the bacteria found from the screening against *P. aeruginosa*. Additionally, 16S rRNA sequencing can be used to determine the exact species of the bacteria found during both screenings. The fruit and vegetable library used in this study can also be used to search for other bacteriocin producing bacteria with activity against other pathogens. Additionally, it could be interesting to further study the nisin Z dominance in the fruit and vegetable samples.

Further research can also be done on the bacteria from the second screening in order to determine if there are bacteriocins that are responsible for the inhibition. If they are shown to be unknown bacteriocins, these can be characterized and tested against other bacteria as well. Additionally, it can be examined whether the inhibition zones observed during screening against *P. aeruginosa* (**Figure 3-8**) are caused by quorum sensing or not.

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A. Appendix

Table A-1 The different greens and sample ID used in this study There are two parallels for each fruit, one with (+) and one without (-) added salt.

Sample ID	Source
1	Green grapes
2	Blue grapes
3	Orange watermelon
4	Small cucumber
5	Sweet cherries
6	Avocado
7	Pineapple
8	Purple aubergine
9	Apricot
10	Raspberries
11	Blueberries
12	Tomatoes
13	Mango
14	Blue plum
15	Ladyfinger/Okra
16	Dates
17	Passion fruit
18	Strawberries
19	Plums
20	White aubergine
21	Physalis
22	Pear
23	Karela/Balsam pear
24	Taro/Eddo Roots
25	Chayote/Chow chow
26	Banana

Sample ID	Source
27	Kelek/Turkish cucumber
28	Romanesco broccoli
29	Sugar peas
30	Aristo
31	Chinese cabbage
32	Quince (Kvede)
33	Fichianindia
34	Rambutan
35	Eddo/Taro
36	Fig
37	Green, small mango
38	Sweet potato
39	Lychee
40	Sharon
41	Aubergine
42	Large chili
43	Kiwi
44	Red onion
45	Blackberry
46	Rotten apples from garden
47	Small, green chili
48	Dragon fruit (Thanh long)
49	Jackfruit
50	Longgong
51	Apples

Table A-2 Primers used in this study. The primers used for the rep-PCR contains the base inosine (I), which base pairs most favorable with the bases A, T and C, although with a bond weaker than A:T (Watkins & SantaLucia 2005).

Primer	Sequence
REP-1R	5'-IIIICGICGICATCIGGC-3'
REP-2I	5'-ICGICTTATCIGGCCTAC-3'
nisZ-R	5'-GGATAGTATCCATGTCTGAACTAAC-3'
nisZ-F	5'-CTACAAAATAAATTATAAGGAGGCACTC-3'

Table A-3 Table showing the ID and origin of the isolated bacteria inhibiting of *S. aureus*. "+" indicates samples with added salt, while "–" indicates samples without added salt.

Sample ID	Origin	Fruit
S1 - S3	6-	Avocado
S4 - S6 D5-D9 D10-D13	8-	Purple aubergine
S7 - S15	27+	Kelek/Turkish cucumber
S16 - S18	29-	Sugar peas
S19	47-	Green chilies
S20 - S25	48-	Dragon fruit
S26 - S30	48+	Dragon fruit
D1-D3 D14-D16 D24-D25	20+	White aubergine

Table A-4 Table showing the ID and origin of the bacteria obtained from the screening against *P. aeruginosa.* The "-" indicates fruit or vegetable sample without added salt, while "+" is with added salt.

Sample ID	Source	Fruit
S39 - S42	34-	Rambutan
S43	35+	Eddoes
S44 - S45	35-	Eddoes
S46 - S47, S54	3+	Orange watermelon
S48 - S50	6-	Avocado
S51 - S53	4-	Small cucumber
S55 - S57	19-	Plums
S58 - S60	20+	White aubergine
S61 - S62	27-	Kelek
S63 - S64	41-	Eggplant
S65	47-	Small, green chili
S66 - S67	48-	Dragon fruit
S68	48+	Dragon fruit
S69	49-	Jackfruit
S70	49+	Jackfruit

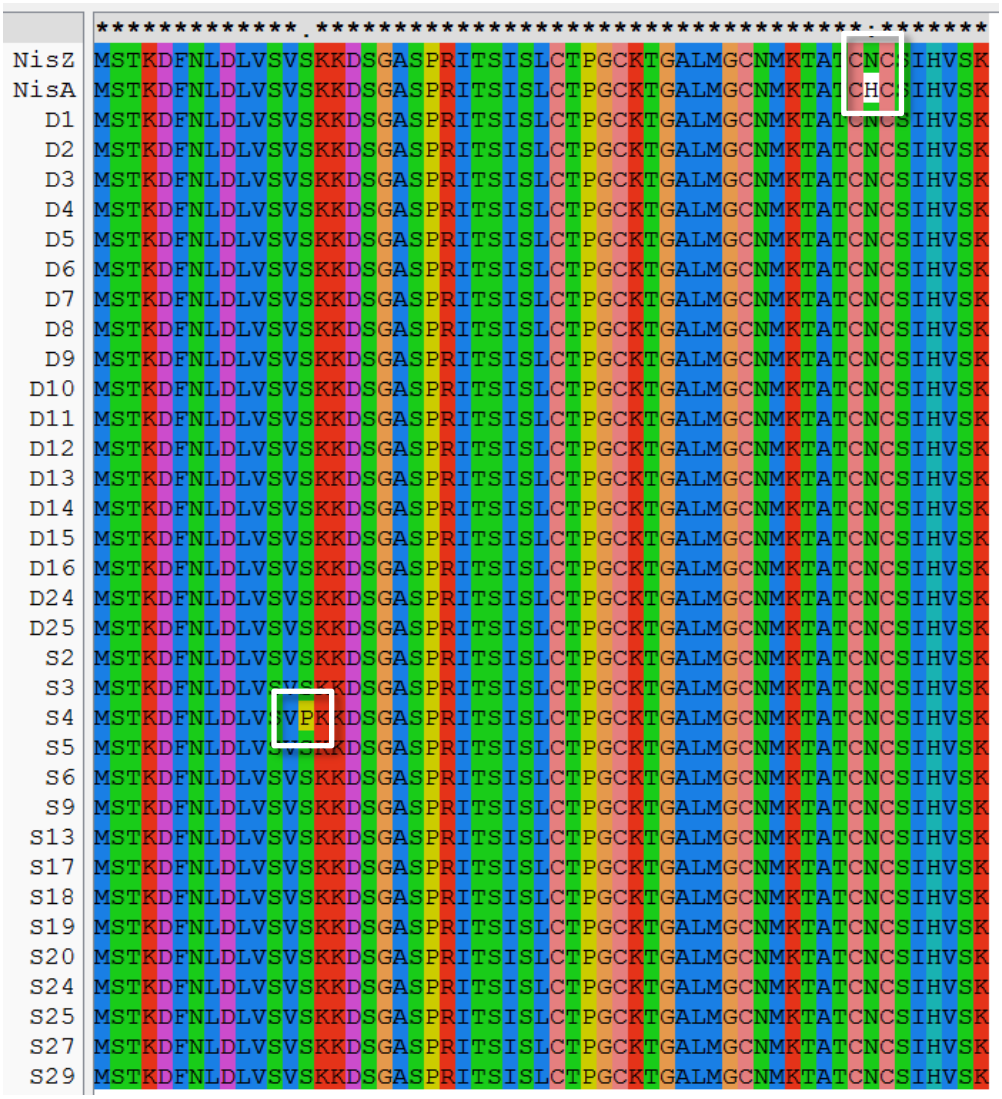

Figure A-1 Pairwise alignment of amino acid sequences obtained by sanger sequencing of the *nis*-gene for all of the sequenced samples. The top two are the sequences for *nisA* and *nisZ* are both obtained from the NCBI database. The different amino acid in S4 is marked by a box, but is in the propeptide region, thus having no biological effect.

Table A-5 Complete API fermentation profiles for the selected bacteria.

Carbohydrate	Sample ID															
	S1	S4	S7	S16	S18	S19	S20	D1	D5	D16	D14	D24	D25	D4	P3	P4
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Erythritiol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-arabinose	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-
D-ribose	+	+	+	+	±	+	+	+	+	+	+	+	+	∓	+	±
D-xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	∓	-	-
L-xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl-βD-xylopyranoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-galactose	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	∓
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	∓	-	-	-	∓	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-mannitol	+	+	+	+	+	+	+	+	±	+	+	+	+	-	+	-
D-sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl-αD-mannopyranoside	-	-	s+	-	-	-	s+	-	s+	s+	s+	-	-	-	-	-
Methyl-αD-glucopyranoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N-acetylglucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amygdalin	±	+	±	±	±	±	+	±	+	+	+	+	+	∓	+	∓
Arbutin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	∓
Esculin ferric citrate	∓	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-lactose (bovine origin)	+	∓	±	±	±	±	±	+	±	±	±	+	∓	-	+	-
D-melibiose	-	-	-	-	∓	-	-	±	-	-	±	±	-	-	-	-
D-saccharose (sucrose)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
D-trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-raffinose	-	s+	-	-	∓	-	-	±	-	-	+	∓	-	-	-	-
Amidon (starch)	±	∓	∓	∓	∓	∓	±	±	∓	∓	±	∓	∓	±	-	∓
Glycogen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gentiobiose	+	+	+	+	+	∓	+	+	∓	±	+	+	+	+	+	+
D-turanose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-lyxose	-	s+	∓	-	s+	∓	∓	s+	∓	∓	∓	-	∓	-	-	-
D-tagatose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

L-fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Potassium gluconate*	∓	∓	∓	∓	∓	∓	∓	s+	∓	∓	∓	∓	∓	s+	∓	∓	
Potassium 2-ketogluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Potassium 5-ketogluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* most of the samples had tiny air bubbles in the well after 24h, and weak reaction

+ positive reaction after 48h, - no reaction after 48h, ± some reaction after 48h, ∓ some (weak) reaction after 48h

s+ (some) reaction after 72h or later not present at 48h

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