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Purification and Characterisation of New Bacteriocins

Kai Arne Hestad
Chemistry and Biotechnology, Molecular Biology

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Abstract

Not long after the discovery of the first antibiotics in the 20th century, bacteria resistant to this treatment has appeared and the spread of their genes can occur in several ways. This development has just increased, and costs associated with it will be very high in both health and economically. Among some emerging alternatives to antibiotics are antimicrobial peptides, some of which are classified as bacteriocins. These are ribosomally translated molecules that creates pores and permeabilises the membranes of other bacterial competitors in the same ecological niche.

In this project's first part, discovery of the receptor used by the already isolated enterocin NKR 5-3B was attempted. Growth and purification of the bacteriocin was done by ammonium sulphate precipitation, cation exchange chromatography and reverse phase chromatography. A spot-on-lawn inhibition assay was done to obtain an inhibition spectrum, and to generate resistant bacterial colonies in the inhibition zones. The few resistant colonies were attempted to make isolates from, but this did not generate strains that had a genetic and stable basis. These were merely adaptive cells, meaning their resistance against NKR 5-3B was a change in the gene expression pattern, and not due to a mutation in the DNA that codes for NKR 5-3B receptors. Resistant mutants were thus not possible to generate, and a comparison between a wild type and resistant mutants to see what genes were missing in the latter, was barred. This indicates that NKR 5-3B, being a circular bacteriocin, might bind to target membranes without the need for a receptor.

The second part of the project aimed to screen for new bacteriocins from samples of raw milk. Several antimicrobial-producers were found and isolated by using layers of soft agar with samples and indicator separated. The producer strains were first subject to genetic analyses to identify species and genetic heterogeneity, and to identify bacteriocin genes. The two most active strains *E. faecalis* and *S. uberis* were characterised and *E. faecalis* produced enterolysin A. *S. uberis* had a high identity with a bacteriocin-like peptide, but later mass spectroscopy gave results indicating a mass of 2213.4 Dalton. This does not fit any known bacteriocin, but the inhibition profile against indicators show similarities with penocin A. The mass suggests something in the higher

ranges of thiopeptides. Future work on this should include Edman degradation, in which the peptide is sequenced to know its identity.

Sammendrag

Ikke lenge etter funnet av de første antibiotika in det tyvende århundre begynte antibiotika-resistente bakterier å dukke opp, og deres gener kan spres på en rekke måter. Denne utviklingen har bare økt i hastighet, og kostnader assosiert med dette vil bli svært høyt både i helse og økonomisk. Blant alternativer til antibiotika er antimikrobielle peptider, hvor noen er klassifiserte som bakteriosiner. De er ribosomal translate molekyler som danner porer og permeabiliserer membranen til andre konkurrerende bakterier i samme økologiske nisje.

I første del av prosjektet er målet å oppdage reseptoren som bruker av den allerede isolerte bakteriestammen enterocin NKR 5-3B. Vekst og rensing av bakteriosinet ble utført med ammonium sulfat utfelling, kation-bytte kromatografi og omvendt-fase kromatografi. Spot-on-lawn assay ble utført for å se inhibisjonsspektrumet, og for å generere resistente bakteriekolonier i inhibisjonssonene. De få resistente coloniene ble forsøkt isolert og tested, men dette genererte ikke stammer med genetisk og stabilt basis for resistens. Disse var adaptive celler, hvor deres resistanse mot NKR 5-3B er en endring i genutrykk og ikke grunnet mutasjoner i DNA som koder for NKR 5-3B reseptorer. Resistente mutanter oppsto dermed ikke, og en sammenligning mellom villtypen og resistente mutanter for å identifisere manglende gener i mutantene, ble hindret. Dette indikerer at NKR 5-3B kanskje binder til målcellenes membran uten reseptorer, noe som sirkulære bakteriosiner kan gjøre.

Andre del av prosjektet tok sikte på å screene nye bakteriociner fra råmelkprøver. Flere antemikrobielle produsenter ble funnet og isolert med lag av soft agar som isolerte prøver og indikatoren. Produsentene ble så underlagt genetisk analyse for å identifisere arter og sjekke genetisk variasjon, samt identifisere bakteriosingener. De mest aktive stammene *E. faecalis* og *S. uberis ble karakterisert, og E. faecalis* produserte

enterolysin A. *S. uberis* hadde stor likhet med et bakteriosin-lignende peptid, men senere massespektroskopi viste en masse på 2213.4 Dalton. Dette passer ikke med noen kjente bakteriosiner, men inhibisjonsprofilen viser likheter med pediojin eller penocin A. Massen indikerer noe i høyere størrelsesorden hos thiopeptider. Fremtidig arbeid på dette bør inkludere Edman degradering, hvor peptider blir sekvensert for avdekke dets sanne identitet.

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1.0 Introduction

Microorganisms have been an integral part of human existence along its evolution, and is thought to have evolved in both a symbiotic and antagonistic manner (Wilber-Rosenberg & Rosenberg, 2008). Furthermore, the symbiotic bacteria have been thought to inhibit potential pathogenesis in humans (Emiley & Rasko, 2014). Although the beneficial consequences of microbe-human interactions are continually being illuminated, the negative consequences of bacterial interactions have been a primary concern. The notions of contagion have been documented since biblical times as a cautionary tale against leprosy, and Koch and Pasteur came with the more concrete findings of pathogenic bacteria in the 19th century (Drasar, 2010). Ehrlich discovered salvarsan against *Syphilis* in 1910 and Fleming found penicillin that inhibited *Sataphylococcus aureus* in 1928. But pathogenic bacteria adapted alongside this, with penicillin-resisting *S. aureus* appearing already in the 1940s. Since this, discovery of new antibiotics, such as tetracycline and vancomycin have been followed by its resistant pathogenic microorganisms (Saga & Yamaguchi, 2009). The mechanisms for evading antibiotic effects are heterogenous, making them hard to treat. This includes production of enzymes or substrate-binding proteins that inactivate the antibiotics, efflux pumps that transport antibiotics out of the bacteria, reduction in antibiotic uptake, and alteration of the antibiotic's substrate (Kapoor et al., 2017: Wencewicz, 2019)

At the present time, it is estimated that with no novel strategies curbing the growth of antibiotic-resistant bacteria, the consequences will be 2.4 million annual casualties from antibiotic-resistant bacteria in the US, Europe and Australis, and a related cost of $ 3.5 billion US dollars (Windels, et al., 2019). Other calculations indicate that pathogens such as *Staphylococcus aureus*, *Listeria monocytogenes*, noroviruses and functionally related food spoilage microorganisms cost up to $ 34.9 billion every year due to illness from ingesting food (Ibrahim, 2019). What all these calculations have in common is the unmistakable message that this is increasingly costly, both economic and in human health and life.

The spread of bacterial pathogens is a composite process. And there is much that can be done to halt cases of infection. However, looking at studies investigating the spread of infections seems to show the absolute necessity for effective antimicrobial treatments. Looking at quarantine and isolation measures, Manguvo and Afuvadze (2015) describes

that in the Ebola outbreak in 2015, 60 % of Ebola related deaths could be linked to burial rites in Guinea. Isolation initiatives from health organisations were actively undermined because it interfered in such cultural rituals. Belief in healing by touching the sick individuals exacerbated the infection rates when these rites were not interfered with. The only viable option for health workers was to ally themselves with religious and cultural leaders in local communities. The take home message is that one cannot rely on quarantine as a singular strategy to halt infections, virological or bacterial. A direct measure to combat infectious agents themselves seem to be an imperative.

In addition to using antibiotics for treating humans, antibiotics are also used in animal food production to curb infections, among other things. Overuse of this can also be a pathway for human pathogens to increase their resistance to antibiotics as well. The risk is that emerging antibiotic-resistant bacteria can be spread to human pathogens by horizontal gene transfer such as by plasmids or transposons (Salisbury et al., 2002; Shousha et al., 2015). This would exacerbate the increase in human pathogens that are hard to treat. An effective alternative to antibiotics is urgently needed. Even if a treatment would only be useful for livestock, this would still benefit humans by reducing the overall amount of antibiotic use, and reducing the possibility of animal pathogens transferring their antibiotic-resistant genes to human pathogens. A ban on antibiotic use in some countries has shown a decreased prevalence of some resistant bacterial strains like *Enterococci* (Hao et al., 2014). This indicates that antibiotic use in livestock has an effect on bacteria of consequence for humans.

1.2 Bacteriocins and antibiotic alternatives

In light of the increasing challenge associated with an excessive use of antibiotic drugs, research into alternatives has been ongoing for some time. Although it would be wrong to assume such technologies could replace conventional drugs now, they nevertheless show promise in their delineated fields of application. They could also be a solution to employ in those situations where antibiotics fail, or as a synergistic supplement to them.

Several technologies offer interventions against infections by pathogens. This includes predatory bacteria that consume gram negative bacteria, and work on biofilms, as well as not showing significant signs of inflammation while working in an organism (Madhusoodanan, 2019). Phage therapy entails viruses that infect and lyse just a narrow

range of bacteria such as *Enterococcus faecium* and *Clostridium difficile.* They are also used in food safety techniques (Lin et al., 2017). CRISPR-Cas9 can inactivate genes for antibiotic resistance in bacteria, and can be introduced through conjugative plasmids to make bacteria vulnerable to antibiotics again (Rodriques et al., 2019).

In addition to the aforementioned technologies, a range of peptides have also shown themselves to be useful for curbing bacterial growth, called AMPs (antimicrobial peptides). AMPs are made on ribosomes and a natural part of the producer organism's defense system. One of the advantages is that AMPs affect some of the important structures of bacteria, and have done impact through evolution with their targets (Maróti et al., 2011). Although the focus here will be on those produced by bacteria, both animals and plants are also a source of peptides capable of antimicrobial action. For example, Pexiganan is a derivative of the peptide magainin II, originating from Xenopus laevis, an African clawed frog. This came into the spotlight after the frog was given a cut and be immersed in bacteria without being infected. Pexiganan is effective against both gram negative and positive, has a broad range of microbes it inhibits, and is highly stable (Gottler & Ramamoorthy, 2009). It has among other things been used in cream-form to treat diabetes-induced foot ulcers that have become infected, and together with the bacterial AMP nisin, its effect is amplified (Gomez et al., 2020).

Bacterial peptides are the focal point of this project, termed bacteriocins. These are a part of the wider category of antimicrobial peptides (AMPs). Their size generally vary between 30 and 60 amino acids (Jack et al., 1995). While antibiotics are synthesized by an enzymatic reaction, bacteriocins are translated by ribosomes. Like phages mentioned earlier, there is a relatively narrow range of bacterial type inhibition for each bacteriocin. Normally the strains that are affected are types related to the bacteriocin-producer. This is presumably due to competition for resources in the same ecological niche. However, some bacteriocins have a wider inhibition spectrum (Li & Rebuffat, 2020).
Even though both gram positive and gram negative bacteria produce bacteriocins, a large portion are represented among lactic acid bacteria (Silva et al., 2018). These are involved in a large amount of food preservation, and are generally regarded as safe (GRAS)

1.3 Types of bacteriocins

The classification system for bacteriocins have been updated and revised alongside the discovery of new ones with unusual synthesis, structures or modes of effect as seen in light of the already established classification system. Klaenhammer (1993) originally divided bacteriocins into four classes. As more bacteriocins were discovered, the classification system has changed also (Cotter, 2012; Nes & Holo, 2000; Ibrahim, 2019). Among the ways to classify bacteriocins is to divide them based on size and post-translational modification and constituting classes of I, II and III (Alvarez-sieiro et al., 2016). This is the system chosen here, as it divides neatly by size and post-translational modifications.

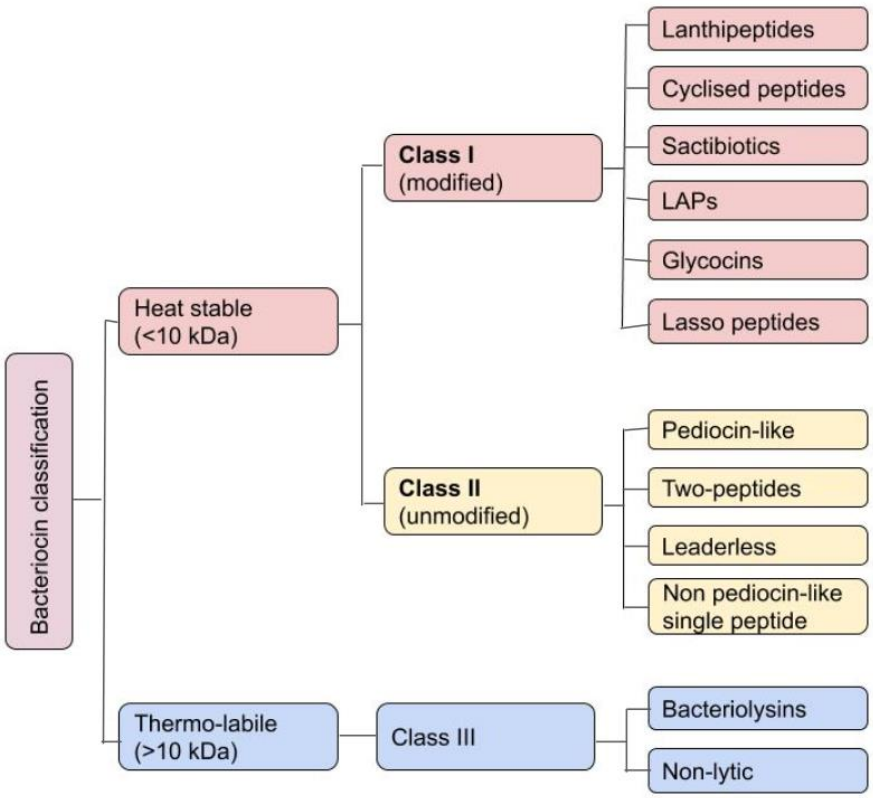


Figure 1.1 *Classification system for bacteriocins, based on Alvarez-Sieiro et al. (2016)*

Class I: Posttranslationally modified peptides (20-35 amino acid residues)

Class Ia

Class Ia consists of the lantibiotics, so named because of their lanthionine or methyllanthionine-containing structure. This characteristic amino acid structure receives its

final form as a result of modification after translation. The genes responsible for enzymes that modify this are located adjacent to each other. Four types of lanthipeptides can be identified based on the types of enzymes that cause post-translational modification, but only two types have observable antimicrobial effect (Alvarez-Sieiro et al., 2016). These peptides have a net cationic charge and inhibits the growth of competing bacteria by binding to receptors. Generally, this category of bacteriocins are the most explored with regards to food storage and production, and cosmetic preservation.

A classic example of this kind is Nisin, produced by *Lactococcus lactis*. It has 34 amino acid residues, and has a broad range of targets that are vulnerable. This includes LAB bacteria, *Listeria*, *S. aureus* and *B. cereus*, to mention a few. It is also considered safe to use as a food preservative (GRAS status). Nisin binds to lipid II in the target cell and creates a pore that makes it leak essential molecules, leading to cell death.

Class Ib

These bacteriocins have a circular shape when they are in their final form. A common trait with the circular bacteriocins is that in their mature form, they have their N-and C-termini joined together by covalent binding. This shape further displays a saposin fold, and the overall form gives a high resistance to proteolysis, pH extremes and heat. Furthermore, these bacteriocins are either cationic or highly hydrophobic (Perez et al., 2018).

The inhibitiory effect against other bacteria class Ib shows is often broad, working against gram-positive bacteria, but not against fungi and gram-negative bacteria. Enterocin AS-48 is one of the typical circularised bacteriocins in this category with a broad spectrum of inhibition (Grande Burgos et al., 2014).

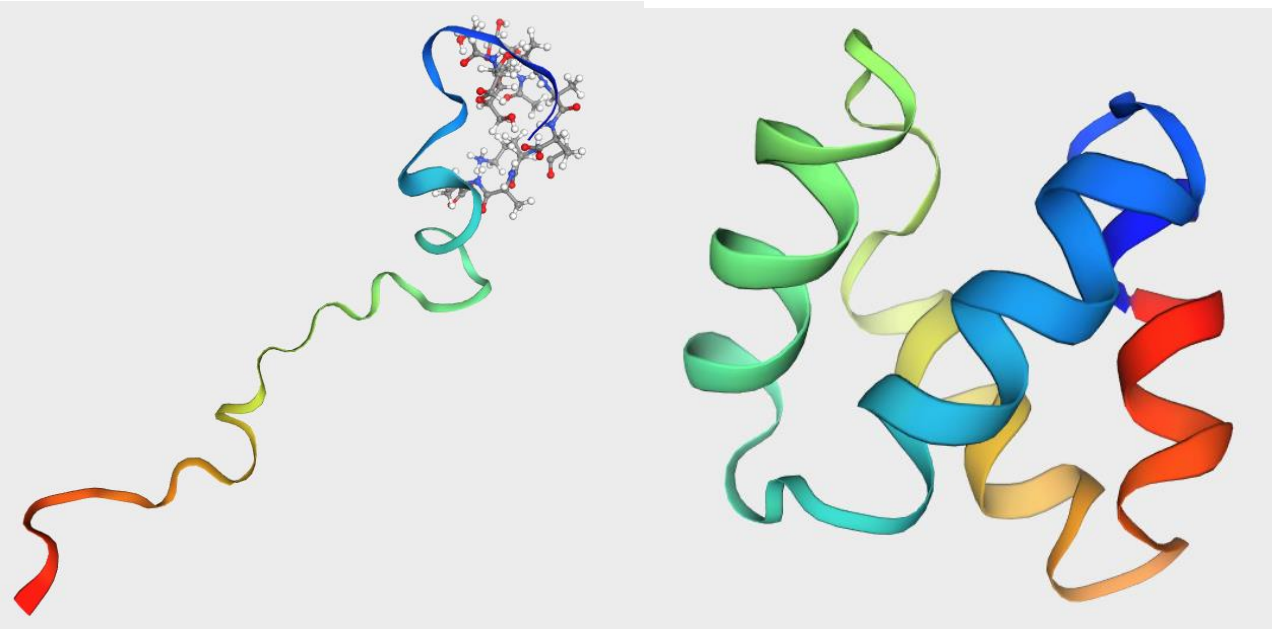


Figure 1.2 *Illustration of nisin a (left) and NKR 5-3B (right). Source: Swiss-model https://swissmodel.expasy.org/*

Class Ic

The characteristic trait of this type of bacteriocin are sulphur molecules bound to the α-carbon in some of their peptides. Subtilosin A, thurincin H, thurincin CD and propionicin F are included in this category. Their shape is often that of a hairpin structure, and they often have the hydrophobic residues of their peptides pointing outwards (Mathur et al., 2017a). This has been theorised as binding to the hydrophobic part of the cell membrane in target cells. Subtilosin A shows this type of action (Thennarasu et al., 2005). Only subtilosin A has a broad inhibition spectrum, the rest are narrow range inhibitors.

Class Id

These bacteriocins are called linear azol(ine)-containing peptides (LAP), and have the unusual amino acids oxazole and thiazole in their peptide backbone. The oxazole and thiazoles have cyclic structures, and are a result of cysteine, serine, and threonine modifications through a series of dehydration reactions requiring ATP (Alvarez-Siero et al., 2016). The LAP streptomycin S belongs in this category. Their mode of action are not well understood, although some LAPs have been explored. For example, phazolicin is a narrow spectrum inhibitor that interacts with ribosomal RNA in the target cell and stops protein synthesis (Travin et al., 2019).

Class Ie

Otherwise referred to as glycocins, these antimicrobial peptides have sugar molecules installed through post-translational gycosylation (Norris & Patchett, 2016). Glycocin F belongs in this class, and its structure contains two peptides that are glycosylated at cysteine, serine and threonine chains, and connected by two disulphide bonds (Amso et al., 2018). Glycocins have two different effects, depending on the species. Some are bacteriostatic, while other types hinders growth by killing the target cells. It is discovered that glycocin F uses a phosphotransferase system transporter as a receptor (Norris & Patchett, 2016)

Class If

These bacteriocins have a circular shape at their N-terminal, giving them the name lasso peptides. An amide at the N-terminal is connected to a fragment seven to nine amino acids further towards the C-terminus, resulting in a circular shape (Hegemann et al., 2015). Microcin J25 is a classic example of this. *Streptococci* and *E. coli* can produce lasso peptides. Interestingly, lasso peptides have widely different effects on other cells, including reducing cancer cell migration in addition to antibacterial activity (Son et al., 2018). There seems to be specific receptors for this type of bacteriocin, siderophore transporters being among them (Rebuffat et al., 2004).

Class II: non-postranslated peptides

The defining traits of this overarching category is the lack of extensive post-translational modification with enzymatic reactions. Beyond this, there might still be leader peptides that need to be removed and disulphide bridges to be connected before the bacteriocin is active.

Class IIa

This class is the pediocin-like bacteriocins. Like the class I, they are cationic, but are larger with 37-48 amino acids. Their general structure are two domains that are attached with a joint between these. Pediocin PA-1 has two disulphide bridges (Oppegård et al., 2015). There is a highly conserved motif of amino acids near the N-terminal part consisting of the amino sequence YGNGVXC. The antibacterial effect is thought to be because of this motif.

In addition, pediocin-like bacteriocins have a broad spectrum of inhibition, and is an excellent inhibitor of *Listeria* strains (Papagianni & Anastasiadou, 2009).

Class IIb

Class IIb bacteriocins are dependent on a synergic effect between two types of peptides to have optimal activity against target cells. Both peptides must be present in roughly similar amounts. Some types of these two-peptide bacteriocins have an effect against bacteria when only one type of peptide is present, but a higher effect is obtained with the addition of both peptides. In the presence of each other, the two peptides form a helical structure that binds to specific receptors and forms pores by insertion into them target cell's membrane (Nissen-Meyer et al., 2009). Studies with Lactococcin G indicated that a protein called UppP, important in construction of peptidoglycan, functioned as a receptor (Kjos et al., 2014).

Class IIc

Leaderless bacteriocins make up this class, and their distinctive characteristics are the absence of a leader peptide that needs to be cleaved off. To make this bacteriocin type more enigmatic, there are no genes for immunity proteins that protect the producing cell. An ABC transporter performs this protective function (Iwatani, 2012). Several of the bacteriocins in this group have similar motifs to some circular bacteriocins in class Ib. These similarities also extend to how they affect target cells. Like circular bacteriocins, these leaderless bacteriocins bind anionic charged membranes without the need for a specific receptor. There are exceptions to this. For example, LsbB employs a zinc dependent membrane metallopeptidase as a receptor to impart its effect (Ovchinnikov et al., 2014).

Class IId

This class is the catch-all category that does not fit into the preceding classes among the unmodified bacteriocins. Its moniker is non-pediocin-like, single-peptide bacteriocins. It contains an array of different linear peptides and the mode of action is equally divergent. Some, like Lactococcin 972, are heat-labile but pH stable, and affects only close relatives to its lactococci producer. This is accomplished by inhibiting the construction of cell walls in the target cell. Lactococcin binds to lipid II and arrests further synthesis. Another well-known member of this category is lactococcin A, which functions by binding to a specific receptor in the target bacteria, namely the Man-PTS system (Diep et al., 2007).

Class III

Class III are larger types of molecules of more than 10 kDa in size. They are also vulnerable to high heat. Often several domains are connected to constitute these bacteriocins in their final form. Two sub-categories exist in class III: the lytic and non-lytic types. The lytic bacteriocins are endopeptidases that include enterolysin A, zoocin A and Millericin B. Bacteriolysins are another name for them. These cut the peptidoglycan structures of vulnerable cells, causing lysis and death. Gram-positive cell walls are the target here (Khan et al., 2013). The non-lytic class III bacteriocins employs a different manner to impede growth of target cells. For example, caseicin will inhibit the synthesis of genetic material in the target (Müller & Radler, 1993).

Other antimicrobial peptides: thiopeptides

There are other peptides with antimicrobial activity as well, thiopeptides being one of these. These are a category smaller than bacteriocins often the size of 1000-2000 Daltons. Structurally they are distinguished by a macrocyclic peptide containing thiazole heterocycles. They are biosynthetically made either ribosomally, or as a result of peptide synthases (Just-Baringo et al., 2014). Actinobacteria er the primary producers of thiopeptides (Kaweewan et al., 2018). They have activity against more than bacteria, such as anti-fungal and anti-cancer effects. In bacteria they inhibit protein synthesis in the ribosomes by blocking an elongation factor G in the ribosome (Just-Baringo et al., 2014). Radamycin is an example of thiopeptides (Rodrigues et al., 2002).

1.4 Biosynthesis and maturation

Although there are many categories of bacteriocins, some common features exist regarding how these are manufactured inside the bacterial cell. Often the genes involved are located together on operons in the DNA of the producer bacteria. Some genes encoding bacteriocins can be located on plasmids instead on chromosomes (Nes et al., 1996). This is dependent on the particular bacteriocin in question.

Since class I bacteriocins are modified after translation of the structural gene, there is generally a need for genes encoding enzymes that facilitate the modification of the pre-bacteriocin. Often there are several operons involved. A precursor or pre- peptide is produced initially, undergoing modifications. Then there are genes responsible for

transport of the bacteriocin out of the cell involved by aid of an ABC transporter. While this occurs, the inactive version of the bacteriocin is further processed by a protease, becoming active as it leaves the producer cell. Nisin is a classic example of this type of class I biosynthesis (Dimov et al., 2005). There is also a specific gene for immunity proteins that confer on the producer some protection against its own bacteriocin (Parada et al., 2007).

The circular bacteriocins in class I are modified by the terminal C- and N- ends being linked by a dehydration reaction, resulting in a circular shape. They are synthesised in a three-step process: as a linear peptide, then a circularization process, before being projected out of the producer cell (Perez et al., 2018). There is a leader peptide localised at the N-terminal, and it is cleaved off after being translated. The leader peptide itself is not necessary for cleavage and circularisation (Gabrielsen, 2013). Circularization as a process seems to require that helical structures with hydrophobic patches are conjoined, but there is no clear findings of the genes for enzymes facilitating this process (Perez et al., 2018). Lactic acid bacteria such as *E. faecalis* and *L. garvieae*, but also a few *Bacillus* and *Clostridium* produce circular bacteriocin types (Gabrielsen et al., 2014). In addition to the biosynthesis gene for a structural protein, there is an immunity gene that produces a hydrophobic protein, a gene encoding an ATPase, and several membrane-related proteins. Findings suggest that several of the membrane proteins are needed for the producing cell to attain full immunity to the bacteriocin, and several are involved in cellular export (Kemperman et al., 2003). Some bacteriocins also have genes for an ABC transporter complex, and it joins the other membrane proteins in enabling bacteriocin immunity by a combined effect.

For class II bacteriocins, the production involves an immature form where there is a leader peptide that is removed at a double glycine site. The leader peptide prevents the bacteriocin from being active inside the producer. An ABC-transporter sequesters these bacteriocins out of the producer cell. The transporter itself also has proteolytic functions, resulting in a mature, active bacteriocin (Nes et al., 1996). There are four genes that always are present to facilitate this type of bacteriocin production: 1) A structural gene generating immature bacteriocin 2) Immunity gene adjacent to the structure gene, 3) an ABC-transporter gene, and 4) a gene for proteins externalising the bacteriocin.

Class III bacteriocins are less studied regarding biosynthesis. But generally, there are genes for structural proteins, transporter proteins, sometimes maturation, and sometimes immunity proteins. Some of the structure proteins, such as in the case of dysgalactin, are located on plasmids (Heng, 2006). Transporters are also identified for dysgalactin, but no post translation corresponding to that of class I bacteriocins (Heng, 2006). In the case of zoocin A, there is maturation, found by the presence of genes encoding an endopeptidase that develops bacteriocins into their active form (Malinicova et al., 2011). In some cases there are examples of immunity proteins being produced as well. Biosynthesis of zoocin A involves production of an immunity protein that gives resistance to the effect of the bacteriocin, namely the cutting of peptidoglycan (Gargis et al., 2009).

1.5 Regulation of production

As mentioned, bacteriocins are ribosomally produced. This requires resources, and it would be illogical for the producer to not have some degree of control over how much bacteriocin to produce and export. The presence of other bacteria is a crucial factor, supported by findings. A three component system of communication follows many types of bacteriocin production regulation. A bacteriocin-like pheromone signal is secreted, and a signalling system detects it. This consists of a histidine kinase protein that autophosphorylates and initiates a DNA activating protein when binding the pheromone (Diep et al., 1995; van Der Ploeg, 2005). This appears to occur within strains, and thus makes up at type of regulation language. Because this is dependent on signaling molecules from other bacterial kin, it is dependent on the bacterial population density (Eijsink et al., 2002). Situational occurrences that impact the concentration will also affect the response, such as degradation of pheromones, diffusion and isolation of the producers (Shanker & Federle, 2017). Some class I bacteriocin producers also employ the produced bacteriocins themselves as pheromone signals received from other bacteria. This is the case for nisin produced by *L. Lactis* (Kuipers et al., 1995) and carnobacteriocin B2 by *Carnobacteriu pisciola* (Kleerebezem & Quadri, 2001).

In addition to quorum sensing and “social effects” between bacteria, the nutrients in the environment and pH will affect the bacteriocin production. It will also indirectly affect it, by influencing the growth of bacteria and their population density (Yang et al., 2018). Temperature also seems to play a role. Experiments with *E. faecium* have shown that a temperature of 35 °C gave maximal bacteriocin production, both by increasing the

bacterial density and bacteriocin production itself (Leroy & De Vuyst, 2002). However, these findings appear to vary between different types of bacteria, and so no general principles can be made for all of them.

1.6 Bacteriocin function and effects

As mentioned, the main function of bacteriocins is a means of competing with other bacteria that the producer shares an ecological niche with. Generally, this involves creating pores and permeabilising the membrane of the target cell, killing it, and thereby reducing the population of competitors. The action of class Ia is to bind to specific receptors, and in the case for nisin Z the receptor is lipid II. Specific parts of the bacteriocin bind to the receptor and another part is inserted into the membrane and forms the pore (Breukink et al., 1999). This causes leakage of ions and loss of charge in the target cell. Disruption of life processes occurs, killing the cell (Abanoz & Kunduhoglu, 2018). The mannose-phosphotransferase system, which is responsible for uptake of some sugars in *Firmicutes* and *Gammaproteobacteria*, can also be a target of bacteriocins. This tends to be utilised by the class II non-modified bacteriocins, such as pediocin-like bacteriocins and lactococcin A. But also the antimicrobial protein microchip E492 produced by a strain of the gram-negative bacteria *Klebsiella pneumoniae* employs mannose permease as a receptor, which is also part of the uptake system for this sugar (Beler et al., 2006; Kjos et al., 2011).

Another mechanism of action is binding the membrane without the need for a receptor, instead relying on charge differences to bind the bacteriocin to the target cell. Class Ib bacteriocins, being the circular type, have a potential for this mode of target disruption. In order to bind without relying on receptors, it is thought that the positive cationic charge facilitates them interacting with the negative charge of target cells (Perez et al., 2018). This binding permeabilises the membrane, resulting in loss of ions and loss of membrane potential, killing the cell (Van Belkum et al., 2011). It was found that subtilosin A reacted generally with synthetic amphiphilic bilayers and exposed the negative charge, inserting itself into the membrane (Thennarasu et al., 2005). This supports the concept of bacteriocins binding to the membrane without the need for a specific receptor. Overall, the circular bacteriocins show a broad range of bacterial strains that they inhibit. If they bind to the target cells' membrane generally, this could be the reason for a broad range of species

that are inhibited. They bind to a basic, general structure that many different types of bacteria share.

Simultaneously, other studies on circular bacteriocins indicate that there might be a receptor-dependent binding operating in parallel with the mechanism described above. This is exemplified by garvicin ML, where the maltose ABC transporter was indicated to be the decisive in sensitivity. Differences between mutants lacking a functional gene for the maltose ABC transporter displayed resistance to garvicin ML. But over a certain concentration of the bacteriocin, the growth of the mutants was also halted, hinting at two mechanisms of action (Gabrielsen et al., 2012). Overall, the circular bacteriocins show a broad range of the strains of bacteria that it inhibits. This fact lends itself to speculation of whether this empirical characteristic is congruent with a general inhibition of bacterial strains. They show a prominent activity against Listeria and Clostridium. But, as indicated, there are several mechanisms of bacterial inhibition that need to be elucidated (Gabrielsen 2014).

Cleaving of peptidoglycan cross-links in the target cell is also a mechanism of action, displayed by zoocin A. Two domains are active, where one side is responsible for binding of substrates, while the other domain targets the bacterial exterior. This lyses the cell, but binding to the cell-wall is essential (Akesson et al., 2007).

A less common effect is by inhibiting synthesis of DNA. Caseicin is a bacteriocin produced by L. casei that is associated with reduced synthesis of proteins and DNA (Müller & Radler, 1993). Sublancin, produced by *Bacillus subtilis*, is also involved in reduced DNA, RNA and protein synthesis, although the exact mechanisms are unclear (Wu et al., 2019).

1.7 Application

Bacteriocins have diverse ranges of usage where bacterial inhibition is needed.
A rather successful area is food preservation. LAB bacteriocins have been very relevant here, as some of them have gained status as GRAS by the FDA (Borges & Teixeira, 2016). There are a few different approaches to preserving food with bacteriocins. The first is to apply LAB in food as a starter culture, and letting them produce bacteriocin in the food as they grow. This is a natural choice for fermented foods. It is also a possibility to add a pre-fermented food component to the dish that should be preserved. Purified bacteriocin

can also be added as a strategy. The latter is deemed impractical due to it being costly and time consuming to purify bacteriocins. The bacteriocins can also be degraded if they are just added to a food matrix, and some types of food might not be applicable for that type of bacteriocin application (Silva et al, 2018). Surfaces of packaging can also have bacteriocins included. Nisin has been used in plastic films to inhibit bacteria at the surface where the antimicrobial compounds gradually are released into the food (Mauriello et al., 2005).

Many medical applications are also possible. Potential treatment for respiratory infection have been a promising area for research. Mycobactereia that can be responsible for development of tuberculosis have shown to be inhibited by nisin and lacticin (Carroll et al., 2010). In addition there is also a connection between existing *Streptococci* in the respiratory tract, where some of them produce bacteriocins, and the capacity to inhibit pathogens such as *Streptococcus pneumoniae* (Santagati et al., 2012). Bacteria responsible for gastrointestinal disease can also be targeted by bacteriocins. By modulating the balance of microorganisms in the gut, bacteriocins can also be used to help treat gastrointestinal disease. In gut microbiota of mice, bacteriocin producers were able to outcompete the pathogenic counterparts of *E. faecalis* (Kommineni et al., 2015). By colonising the microbiota of the gut, such bacteriocin-producers can work as probiotics and thereby improve the immune system of the host (Lopetuso et al., 2019). There are also some bacteriocins with activity against human tumor cells, such as colicin that influence the apoptosis process of such tumors (Chumchalova & Smarda, 2003). This does appear to have promising in vivo results, but at this time only on mice (Arunmanee et al., 2020). Numerous other areas are being explored with regards to bacteriocins therapeutic potential, such as skin disorders, dental therapy and even contraceptive functions (Ahmad et al., 2017). Other medical uses are as an additive in combination with conventional antibiotics (Mathur et al., 2017b). Of course, the medical applications of bacteriocins are not limited for humans. Especially animals for food are of interest, and there are both uses as probiotics in aquaculture and treatment of livestock (Ben Lagha et al., 2017; Desriac et al., 2010).

1.8 Mastitis and bacteriocins in milk

One consequence of antibiotic resistant proliferation of bacteria is the invasion of pathogenic bacteria in cow udders, affecting one major source of food for humans. Overall,

the relevance of mastitis is a reduction in milk production, the suffering of dairy cows, and costs associated with trying to treat or prevent the condition itself. While some of this cannot be quantified in numbers, it has been estimated that mastitis globally costs €16-26 billion, assuming a 271 million dairy cow population. This amounts to a €61-97 per cow (Thomas et al., 2016). Mastitis is caused by a defined set of microorganisms. Heikkilä et al. (2018) lists the major contributors to mastitis to be *S.aureus, Staphylococci, E.coli, C.bovis, S.uberis* and *S.dysgalactiae*. It is of note that many of these are types of bacteria involved in bacteriocin production. Bovine mastitis is caused primarily by *S.aureus* and *S. agalactiae*, and *S. uberis*, respectively. Environmentally caused mastitis, which can be start without milking, has been found to be harder to reduce (Wente et al., 2019).

The lactation phase is when losses of milk production could be most significant. Mastitis can also be subclinical or clinical. Subclinical mastitis ranges between 20-50% of all cases (Zhao & Lacasse, 2008). Mastitis itself involves the inflammation of the mammae, and this reduces the milk yield overall. This is due to the secretory cells being damaged by the inflammation, causing necrosis or apoptosis type of cell death. Most of the bacterial infections cause some degree of reduction in lactatory cells, thus reducing milk yeld. Some of the damage to milk producing cells is caused by the body's own defenses, neutrophils that have a tissue damaging effect. This is thought to occur via reactive oxygen metabolite generation, and enzymatic reaction. These are also brought about as a response to the invasion of pathogens (Zhao & Lacasse, 2008). The duration of the inflammatory state can vary between short-lived to the entirety of the lactation phase.

In the study done by Heikkilä et al. in Finland, (2018), *Staphylococcus aureus* accounted for over 25% of the mastitis cases, while *E. coli*, *S. uberis*, *S. dysgalactiae* and a few other species each constituted a little more than 5% of the cases each. *S. aureus* seems to be a major culprit with a potent pathogenic effect due to toxin production. *E. coli*, while contributing only to 5% of cases, nevertheless has the severest effect when it does cause mastitis. This is due to endotoxin production, which causes swift and severe inflammation. This in turn harms the cells involved in milk secretion. A worst case scenario can develop an *E. coli* infection into loss of all milk secreting cells and death of the cow itself (Burvenich et al., 2003). *S. dysgalactiae* often causes subclinical mastitis along with *S. uberis*, but the latter is more pathogenic and can often result in recurrence. Their effect on lowered milk secretion is less severe than *E.uberis*. *C. bovis* is considered a minor pathogen. *Staphylococci* that are not *S. aureus*, while being prevalent at 46 %, also contributed

negligible to the severity of infections and loss of milk production, as opposed to the toxin-producing *S. aureus*. Together, the previously mentioned bacteria make up the majority of strains involved in bovine mastitis. Given that a significant part of these are bacteria that potentially produce bacteriocins, it would be useful to harvest and characterise bacteriocin-producing members of these species that potentially could be used to treat or prevent mastitis.

1.9 The purpose of the study

The aim in this project is twofold. Part one involves an already discovered bacteriocin from the producer *Enterococcus thailandicus* (B1724). The bacteriocin is called thaiocin 1, or enterocin NKR 5-3B (NKR 5-3B for short). This has been characterised by previous work at the lab (Hololovina, 2018). In order to elucidate what the mode of action is with this circular bacteriocin, mutants will be generated from inhibition tests. The mutants, once isolated, will be compared to the wild type of the same strain of target cells by genetic sequencing. This is done in order to identify the common denominator that is missing in the mutants, but present in the sensitive wild type. Introduction of the missing gene in a mutant strain could test a causal link between the gene for the cellular structure in question and the sensitivity to the bacteriocin.

The second part of this project revolves around the discovery of novel strains of bacteriocin producers. Samples of raw milk have been supplied by the KMB department of Dairy Technology and Food Quality located at the University. The samples will be screened to check whether they inhibit the growth of specific bacteria, called indicators. The bacteria producing antimicrobials will be isolated and subjected to a series of analyses and purification to characterise it. In the flowchart below the procedures for part 1 and 2 will be outlined.

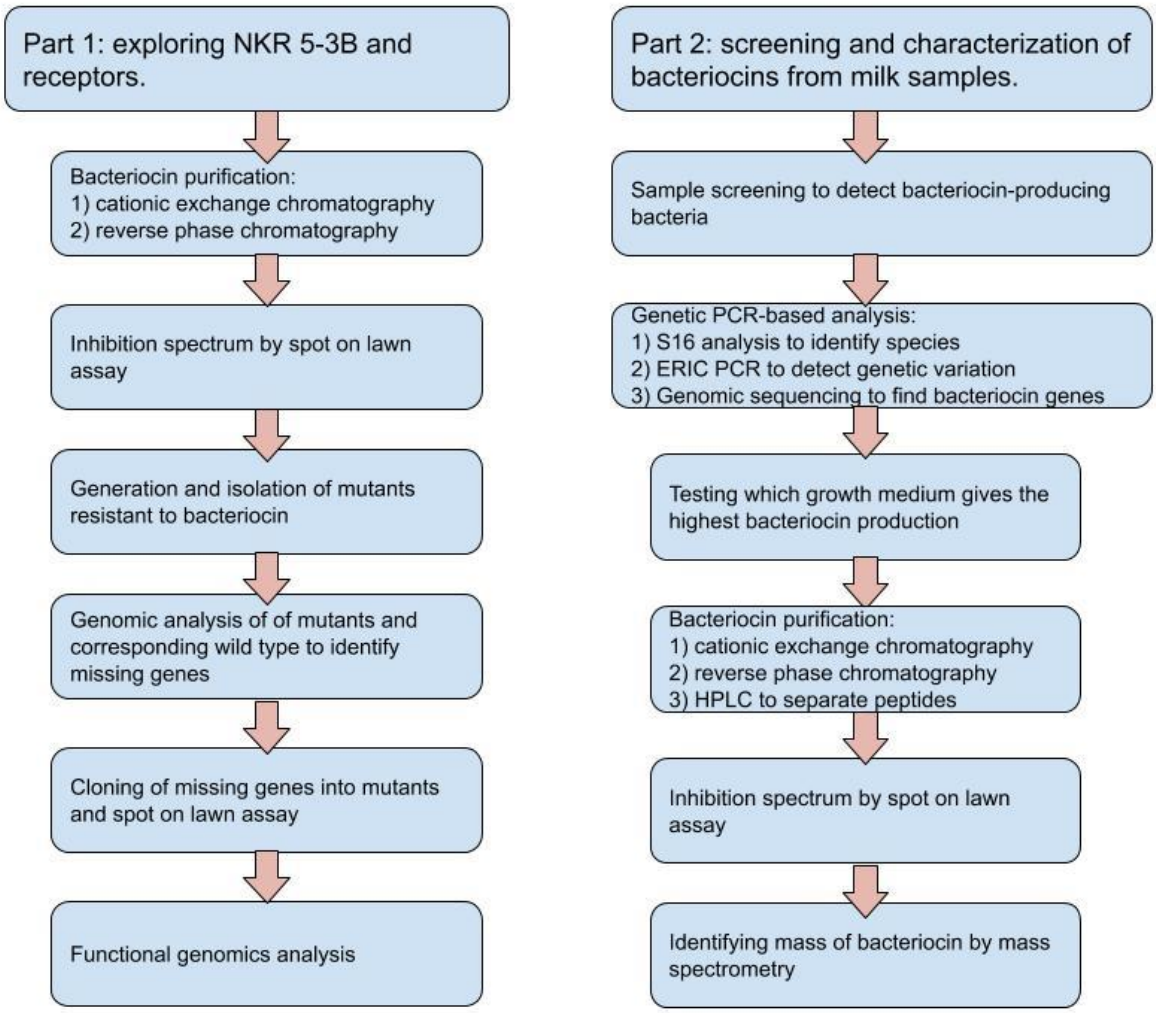


Figure 1.3 *Flowchart of the work process for the project's different phases.*

2.0 Materials and methods

As described earlier, this project has two parts. Part 1 deals with exploring receptors for the specific bacteriocin enterocin NKR 5-3B. For brevity it will be referred to as NKR 5-3B. In part 2 raw milk samples are screened, and its findings are characterised using different

techniques. Several protocols for obtaining results have been used in the study. Some methods have been used in part one, and not in part two, and vice versa. In order to create an overview, figure 2.1 lists the methods that have been employed in which part of the project.

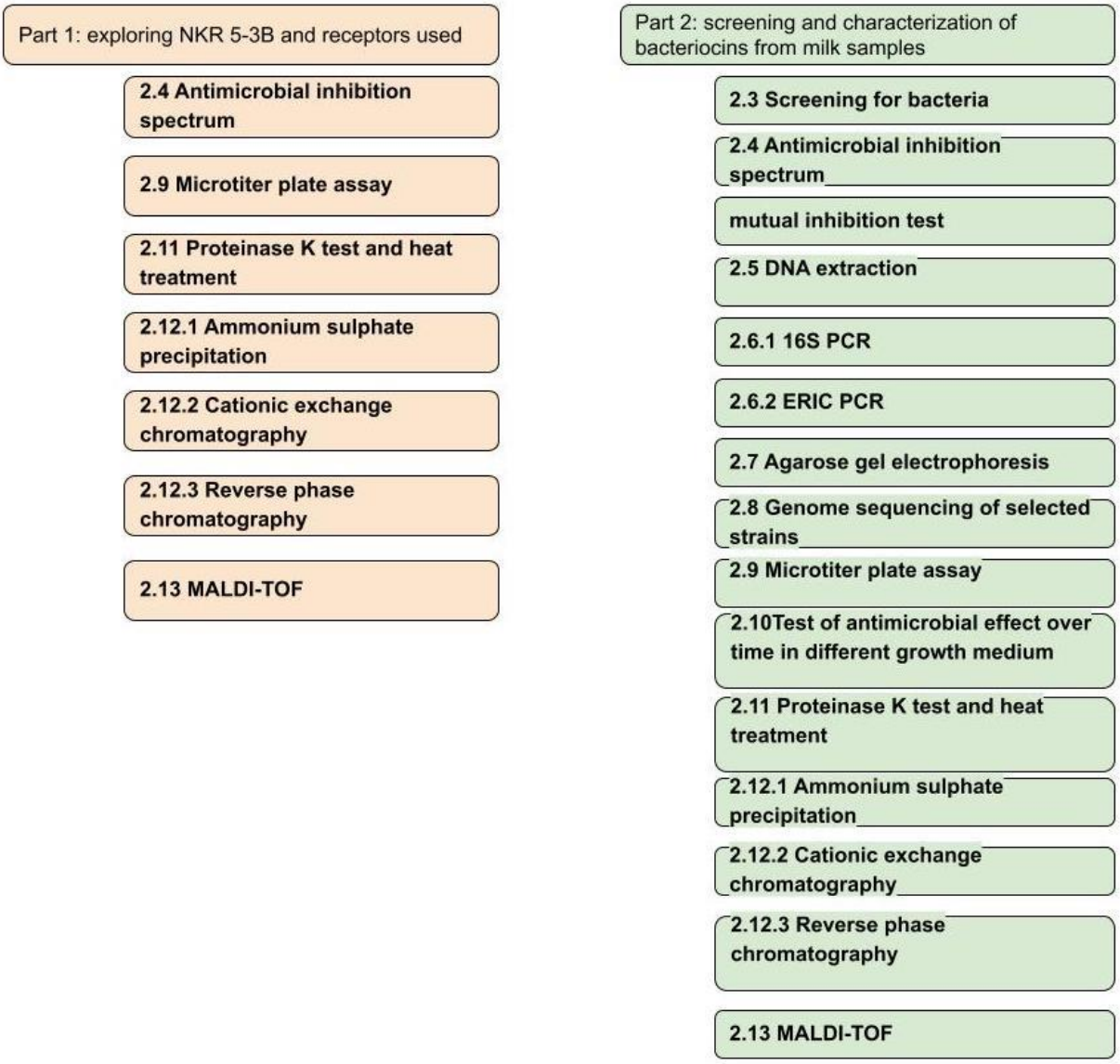


Figure 2.1 *Overview over lab techniques for the two parts of the thesis.*

2.1 Source of bacteria

Two approaches to cultivating bacteria have been used. In the first part, an already isolated bacterial strain of *Enterococcus thailandicus* has been supplied. This has been identified in a previous master thesis conducted at the LMG laboratory (Hololovina, 2018). The thesis' other part has used bacteria isolated as a part of this project. The samples from where the bacteria are isolated have been supplied by the KMB laboratory of Dairy Technology and Food Quality.

2.2 Culture media

A variety of bacteria have been used in the study, and thus their growth media have been used to optimise their growth. Brain, heart infusion medium (BHI) (OXOID), de Man, Rogosa and Sharpe (MRS) (OXOID), M17 (OXOID), and Todd-Hewitt (TH) (OXOID) have been utilised as growth medium, as instructed by the information supplied by the manufacturer. GM17 was made by adding to M17 glucose for a concentration of 0.5% (w/v) glucose in a sterile bench after autoclaving. As needed, these mediums have been used in the form of liquid broth, soft agar and solid agar. To make soft agar, 0.8 % agar was used, and a 1.5 % was mixed in to create solid agar. All types of medium were autoclaved prior to use. A sterile bench and utensils handled with disposable gloves was used when avoiding contamination was essential.

2.3 Screening for bacteria

The screening for bacteriocin-producers was done on the 1 ml samples of raw milk supplied by the KBM department for Dairy Technology. The method is an arrangement of soft agar layers illustrated in figure 2.1. First, a dilution series ensured that there was a variety of bacterial colony densities to choose from. 330 µl of pure sample was allocated to

an Eppendorf tube. 30 µl was then transferred from the first Eppendorf to a new tube and filled to the 300 µl mark with 0.9 % saline solution. From the second tube a new 30 µl was transferred to a third one, followed by mixing with saline solution to a total of 300 µl. This led to a series of three dilutions to work with.

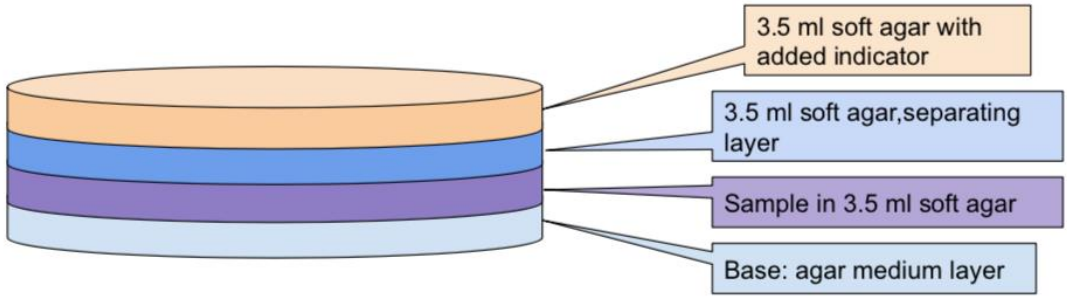


Figure 2.1. *Schematic of different layers in the screening procedure.*

GM17 agar plates were used as the bottom layer in the sandwich overlay. On top of this, 300 µl of the sample was dissolved in 3.5 ml of molten gm17 soft agar kept at a 45°C water bath, followed by a swift vortexing. This was then quickly spread on top of the solid GM17 agar layer, making up the second layer. After solidification, a 3.5 ml of molten 45°C of pure GM17 soft agar was added, constituting the third layer. The purpose of this was to keep the bacterial colonies of the second layer and what was to be added as an indicator separate from each other. The agar plates were then incubated at 30°C under aerobic conditions overnight.

The final layer was added the following day, consisting of GM17 soft agar mixed in with an indicator in the ration 100 µl of the indicator for every 5 ml of soft agar. This mixture was vortexed, applied and spread evenly by a pipette boy. 3.5 ml was distributed to every plate. This was allowed to be incubated according to the optimum growth temperature for the indicator. In some plates there were inhibition zones, and the bacteria from the second layer presumed to be producers of the antimicrobial substance were picked with a toothpick and used for more study.

The milk to be screened was 53 different 1 ml samples of raw milk. Two different approaches for indicator strains to use in the third layer of agar were used. Indicator strains for the first 28 of milk samples were *Enterococcus faecalis* (B3330) and *Staphylococcus aureus* (B3325), mixed together in equal amounts. The samples of raw milk was screened with three dilutions each, generating 84 plates. The combined indicators approach was abandoned, instead opting for using only a single indicator at a time, to obtain an optimal amount of inhibition zones in the screening process. This was done for the remaining 25 samples. Each sample of raw milk allowed for three indicators each: *Streptococcus dysgalactiae* (B3890), *Staphylococcus aureus* (B3023), and *Enterococcus faecalis* (B2333). With three different indicators and three dilutions each, this made 225 agar plate screenings.

After inspecting the inhibition zones from the first round of screening, a sterile toothpick was employed to impale the bacteriocin-producing colony. This was spread onto a new GM17 agar plate to acquire single colonies. After making sure the colony originated from a single bacteria, this was again tested against the original indicator to make sure it's the original source of that colony. If this was the case, it was isolated and put in 15% glycerol and stored at -80 °C.

2.4 Antimicrobial inhibition spectrum and mutual inhibition test

An integral ability of the bacteriocin is its ability to kill or inhibit certain strains of bacteria. As mentioned, the range of different species it can affect, can be narrow or wide depending on the bacteriocin produced. This technique was applied to the bacteriocins produced by *E. thailandicus* and *S. uberis*, 42 and 18 different indicators, respectively.

Indicators were prepared overnight by incubated in BHI broth at 30°C, while the bacteriocin producers were grown in temperatures according to their optimal growth temperature. *E.thailandicus* at 30 °C in MRS, *S.uberis* in BHI at 37°C. After 24 hours, 100 µl of each indicator was mixed in 5 ml of soft BHI agar and poured over a BHI agar plate. After this was set, 3 µl of the purified bacteriocin or bacteriocin-producing culture was applied in distinct zones. For the agar plates with NKR 5-3B being tested, nisin A and garvicin ML was used as a comparison. With the agar plates were the antimicrobial from

S.uberis was tested, nisin A was used as a comparison. The agar plates with indicators and applied antimicrobials were allowed to grow aerobically at 30 °C to the next day.

A special variant of this technique was applied to the 13 samples chosen in part 2. Here, each of the samples were employed as an indicator, each on its own GM17 plate, while the other samples were tested to see if they inhibited indicator growth. They were applied in 3 µl drops. For the Enterococci and Streptococci, the growth temperatures were 30 °C and 37 °C, respectively. Besides these changes, the mixing was as for the inhibition spectrum.

2.5 DNA extraction and NanoDrop

Two methods of DNA extraction was used, depending on the requirements of the quality of the genetic material. For the 16s rRNA analysis, an extraction using a E.Z.N.A ® plasmid DNA minikit (Omega Bio-tek) was used.

The first step was to isolate the cells from the 5 ml overnight culture grown in GM17 by centrifuging it at 10 000 x g for one minute. The pellet of cells was kept and mixed with 250 µl of solution I with RNase A, followed by vortexing to mix. This solution regulates the pH most ideal for cell lysis. It contains EDTA which keeps DNases from working, while RNase A deteriorates RNA. To lyse the cells, the samples were moved to fast prep tubes and glass beads (Sigma-Aldrich) of size 106 µm was added, about 0.5 g to each of the 13 samples. A FastPrep® 24 apparatus (MP Biomedicals) treated the samples at 4 m/s for 3 rounds of 20 seconds each, cutting the cells and releasing the genetic material into solution. After centrifuging to pellet the glass beads, the solution was sequestered from the beads into fresh Eppendorfs. 250 µl of solution II was added to each sample and inverted gently, followed by incubation for 3 minutes. Soltion II denatures DNA and proteins with SDS and NaOH. 350 µl of Solution III was then added and upturned until a a white precipitate appeared. After centrifugation at 13 000 x g for 10 minutes, the supernatant was carefully transported into an assembled HiBind DNA Mini column and 2 ml Collection Tube. Centrifugation at 13 000 x g for one minute gave a filtrate that was discarded, followed by 500 µl HBC and isopropanol buffer added. The column was then centrifuged like previously for one minute, and the new filtrate was removed again. Next. 700 µl of a DNA wash and ethanol buffer was pipetted into the column and it was then centrifuged at 13 000 x g for 30 seconds, this buffer and centrifuge step repeated once. To evaporate any ethanol the column was centrifuged at maximum speed for 2 minutes and the HiBind

column was put in a new eppendorf tube. To this was added 50 µl deionised water and incubated for one minute. Finally, the column was centrifuged at highest speed for 1 minute, and the eppendorf was stored at -20 °C.

Sigma's GenElute® Bacterial Genomic DNA kit provided DNA extraction of a higher quality for the whole genome sequencing and the ERIC PCR. Overnight cultures of 1.5 ml were initially spun down at 13 000 x g for 2 minutes, followed by removal of the supernatant. Each sample was then added 200 µl of lysozyme solution at 45 mg/ml concentration with 250 u/ml of mutanolysin added. A 37 °C incubation was undergone for half an hour. Then 20 µl of an RNase A solution to inactivate RNases was applied to each sample. After 2 minutes of room temperature incubation, a 20 µl prepared 20mg/ml Proteinase K solution was added, mixed, and then a prepared lysis solution C was put in. 200 µl of this was applied to each sample and mixed well. 10 minutes of 55 °C incubation followed, and 13 binding columns with collection tubes were prepared with 500 µl each of a Column preparation solution to increase the DNA binding. This was centrifuged at 12 000 x g through for 1 minute, then removing the liquid. Afterwards, a 200 µl 96% ethanol was added to the lysed samples and mixed well to ensure homogeneity. This precipitates the DNA that has escaped the lysed cells. Subsequently the contents were passed carefully into the prepared columns and centrifuged at 7000 x g for 1 minute, moving the binding column to a new collection tube. Wash solution 1 was added, 500 µl to each sample was washed and centrifuged again at 7000 x g for 1 minute. A new round of a wash concentrate at similar amount to the previous wash was added, followed by 3 minutes of 13 000 x g centrifugation. Another 1 minute of similar centrifugation followed after discarding the flow trough. The binding columns were again moved to fresh collection tubes, followed by 200 µl of nuclease-free water to each column. These were allowed to incubate for 5 minutes. Finally, this was centrifuged at 7000 x g for 1 minute, resulting in eluted DNA. This was stored at 4 °C.

NanoDrop measurement was then used to measure the purity of the extracted DNA. For this a NanoDrop ND-1000 (NanoDrop Technologies) was used. To assess DNA an absorbance wavelength of $\lambda = 260$ nm would absorb the structures of nucleic acids. A detector would measure the concentration of DNA based on this absorbance.
In addition, the purity could be measured by the values of several ratios of absorbance. Purity of DNA was indicated by the A 260/280 ratio, where a value of 1.8 is optimal for pure DNA. Lower values than this could mean protein contaminants. Presence of EDTA,

phenols or carbohydrates could be the case if the A 260/230 ratio is low, as these molecules absorb at λ= 230 nm. A high 260/230 value could be a sign of faulty blank measure, due to a dirty measuring pedestal or a wrong type of blank solution.
Proteins can also be measured by the NanoDrop measurement, but a λ= 280 nm would be chosen as wavelength due to proteins showing absorbance at this frequency.
Before the reading, a blank was used first. This solution should be identical to the sample one wants to measure, except it should be lacking the substance one wants measured. In effect, only the substance of interest should differ between the sample and blank. After reading the blank, one can measure the solution with the protein or DNA to obtain the data mentioned above.

2.6 PCR (Polymerase chain reaction)

In order to have a sufficient concentration of genetic material for proper analysis, the initial amount of DNA must therefore be amplified through the polymerase chain reaction (PCR). Depending on the purpose, some sections of isolated DNA will be subject to amplification. This is done by using specific primers described under each technique.

2.6.1 16S rRNA PCR (polymerase chain reaction)

In order to identify the species in the samples of raw milk that showed inhibition potential, the DNA isolated from the E.Z.N.A ® plasmid DNA minikit (Omega Bio-tek) in section 2.5 was used. The 16S technique exploits the conserved ribosomal sequences that over evolutionary time have changed sufficiently to enable differentiation according to relatedness between bacteria. This allows for identification of bacterial genera in the samples that have been isolated.

In order to amplify the products before 16S analysis, specific primers were used: 16S-12R (5'-AGGGTTGCGCTCGTT-3') and 16S-11F (5'-TAACACATGCAAGTCGAACG-3'). A master mix was prepared with nucleotides, the primers, a thermo stable polymerase and a OneTaq buffer. This was distributed to PCR strips up to 23 µl in each well. This was topped with 2 µl of template DNA extracted as described above. After vortexing and spinning down, the PCR strips were positioned in an A S1000 TM thermal cycler (Bio-Rad). The temperature variations to run the PCR reactions were programmed according to table 2.2

***Table 2.1**. Master mix components for the 16S rRNA PCR*

PCR component	µl for single sample	µl for 13 samples
5X OneTaq buffer	5	65
10 mM dNTPs	0.5	6.5
10 µM 16S-11F primer	1.25	16.25
10 µM 16S-12R primer	1.25	16.25
OneTaq DNA polymerase	0.25	3.25
dH_2O	14.75	191.75
Total volume	23	299

***Table 2.2** The program of thermocycles to facilitate the 16S rRNA PCR*

PCR process	Temperature	Processing time	Cycles
Starting denaturation	94 °C	5 min	1
Continued denaturation	94 °C	45 sec	34
Primer annealing	58 °C	1 min	
Primer extension	72 °C	1.5 min	
Final DNA extension	72 °C	6 min	1
Storage temperature	4°C	Until removed	Nil

After completion of the PCR, the products were mixed with 50 µl of NTI binding buffer. This was then centrifuged 10000 x g for 30 seconds after each sample being placed in a collection tube. Flowthrough was discarded, the remaining DNA was washed with NT3/alcohol buffer, 350 µl for each sample. The products were then centrifuged at maximal speed for 1 minute to remove NTI buffer. The DNA was then eluted with NE buffer 20 µl for each sample. After making sure the DNA concentration was around 50 ng/µl by dilution, by using NanoDrop measurement, two tubes of 5 µl of each sample was

prepared, and each was mixed with one of the primers. It was then sent for sequencing at GATC Biotech.

2.6.2 ERIC (Enterobacterial Repetitive Intergenic Consensus)-PCR

After identifying the species by 16S rRNA PCR, analysing eventual diversity between members of the same species was prudent. This might affect which samples subsequently were chosen for further characterisation. Enterobacterial Repetitive Intergenic Consensus (ERIC) Polymerase Chain Reaction (PCR) was employed. This is because the ERIC primers have been demonstrated to successfully differentiate the relatedness between the species identified by 16S rRNA PCR analysis from the raw milk samples (Jurkovič et al., 2007; Matsumoto et al., 2001). ERIC sequences are intergenic repetitive palindromic units that are conserved and widely dispensed among a wide variety of prokaryotes. The sequences can be detected and display a unique fingerprint according to the presence of these in different strains of prokaryotes. By using primers that are complementary to the sequences of interest, the polymerase chain reaction can amplify these sequences. The resulting bands from this can be visualised by using agarose gel electrophoresis (de Brujin, 1992).

The preparation for the PCR required specific ERIC primer pairs with the following sequences: ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3'), and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'). Together with a thermostable DNA polymerase, this will initiate DNA synthesis. All the necessary constituents except for the extracted DNA were mixed in a master mix in table 2.3. PCR strips were filled with 18 µl for each well with the master mix. 7 µl of each sample's template DNA was added to one of the wells in the PCR strips, resulting in 25 µl total for each well. A S1000 TM thermal cycler (Bio-Rad) was then used to facilitate the PCR reaction itself, according to the protocol in table 2.4. Functionally, the temperature is raised to initiate denaturation and separation of the DNA strands. Then, cycles of maintaining the denaturation, annealing of primers and the existing strands, initiation of the polymerase and its synthesis of new DNA strands, and then elongation of DNA commences in cycles. Finally, the bases are allowed to maximally extend and the DNA to reanneal. The PCR program will keep the temperature at 4 °C, which is optimal for short-term storage.

Table 2.3 *Contents of ERIC-PCR master mix*

PCR component	µl for single sample	µl for 13 samples
5X OneTaq buffer	5	65
10 mM dNTPs	0.5	6.5
10 µM ERIC1R primer	1.25	16.25
10 µM ERIC2 primer	1.25	16.25
OneTaq DNA polymerase	0.25	3.25
dH_2O	9.75	126.75
Total volume	18	234

Table 2.4 *ERIC PCR thermocycler program to produce enough genetic material*

PCR process	Temperature	Processing time	Cycles
Starting denaturation	95°C	7 min	1
Continued denaturation	94°C	1 min	30
Primer annealing	52°C	1 min	
Primer extension	65°C	8 min	
Final extension	65°C	16 min	1
Storage temperature	4°C	Until removed	Nil

The next day, the PCR products were subjected to a gel electrophoresis procedure, see section 2.7. The resulting gel product with bands was then placed in a molecular imager ® Gel doc TM XR + System (Bio-rad) and an image was obtained.

2.7 Agarose gel electrophoresis

In order to differentiate DNA fragments according to size, a gel electrophoresis technique was used. When being applied to wells in the gel, electricity is applied to the gel which leads the negatively charged DNA to migrate towards the cathode direction. The agarose matrix in the gel will hamper DNA migration, with bigger fragments moving slower than smaller ones. This will create distinctive bands that can be observed by UV light and staining the DNA with fluorescent dyes.

To make the gel 1.8% agarose (0.9 g) was mixed into 50 ml 1X TAE buffer, heating this by microwave to solvate the powder. 2 µl Peqgreen dye was then applied to the gel mixture. This stains any DNA or RNA in the electrophoresis, and makes them fluorescent when struck by UV light. The mix was now transferred into a mold, and a comb was inserted at one end of the mold. This was left to solidify for 40 minutes, after which the comb was removed, leaving wells for application of PCR products. The gel is placed in an electrophoresis chamber and immersed in 1X TAE buffer. A 1 kDa ladder (2 µl ladder and 6 µl loading buffer) was applied to the first well, while the remainder of the well were loaded with the samples (10 µl sample with 2 µl loading buffer). The voltage applied for the gel is 70V for 120 minutes for the DNA bands to move sufficiently. A S1000 TM thermal cycler (Bio-Rad) subjected the gel to UV rays and documented the bands along with the ladder as a standard.

2.8 Genome sequencing of selected strains

Based on the results of the 16S rRNA analysis, 6 samples were chosen for sequencing, and 2 were chosen from each species that were more than one type.

Table 2.5 *Overview over bacteria chosen for genomic sequencing*

Sample number	**Species**
#8	*Streptococcus uberis*
#13	*Streptococcus uberis*
#30	*Enterococcus faecalis*
#43	*Enterococcus faecalis*
#269	*Streptococcus dysgalactiae*

#451	*Streptococcus parauberis*

The samples would be analysed by Davide Porcellato, using a Miseq (Illumina) platform for whole genome sequencing. The data obtained from the sequencing would be in the FASTQ type files. After being converted to FASTA format, these would be annotated on the RAST server (Rapid Annotation using Subsystem Technology). This allowed for web-based programs to analyse the DNA data.
BAGEL (BActeriocin GEnome mining tooL) contains catalogues of potential bacteriocins already discovered. Uploading the FASTA files on the BAGEL site generates hits if the query sequences matches the database catalogue. A BLASTp (Basic Local Alignment Search Tool, Proteins) search would then be performed to check if this matched the databases. On basis of this the soundness of the gene being real would be evaluated. Any hits from the BAGEL site would be inquired further by BACTIBASE which contains further information on the bacteriocins in question.

2.9 Microtiter plate assay

This is a technique used to effectively assess to what extent relevant bacteriocin-producers could inhibit indicators in different growth media and differing conditions. This was done on 96-well Sarstedt microplates with micropipettes and multichannel pipettes (Thermo Scientific). The first column was added 100 µl filtered supernatant (or 10 µl bacteriocin solution and 90 µl medium broth in the case of purified bacteriocin). To wells in column 2-10 was added 100 µl growth medium and well 12 was added 200 µl medium. By using the multichannel pipette, 100 µl growth medium was suspended into the tips and used to perform a two-fold dilution series starting with column 1 and working up to column 10 by pipetting up and down 10 times in each column. An indicator that would be inhibited by the antimicrobials in the supernatant was prepared from overnight growth and diluted 25 times in growth medium broth in a falcon tube. Then 100 µl and 200 µl of the indicator was added to columns 1-10 and 12, respectively. This was then incubated at the optimal growth temperature for the indicator for 3-4 hours, or until the indicator had shown sufficient growth. After this time the Sarstedt microplate was read in a SPECTROSTAR Nanoplate reader (BMG labtech).

2.10 Test of antimicrobial effect over time in different growth medium

In order to produce a maximal amount of antimicrobial substances for the purification part of the project, a crude investigating into which growth medium best facilitated this was conducted. The inoculation of bacteria was done with a toothpick without measuring exact starting OD, in order to best mimic how the inoculation of bacteria for bacteriocin purification is done.

This experiment was done for the antimicrobial peptide producers in part 2, because thaiocin 1/ NKR 5-3B has already been tested for this (Holivololona, 2018). The *E. faecalis* producer in sample #43 was grown in BHI, GM17 and MRS medium, while the *S. uberis* producer of sample #13 was inoculated in BHI, GM17, MRS and TH (Todd-Hewitt) medium. The latter has been devised particularly for cultivating streptococci species, so it was deemed fruitful to explore this as a medium for bacteriocin production from this species as well. Three points of time were chosen to collect 1.0 ml aliquots for each species in each medium, filtered by 0.45 mm filters, heated at 95 °C for 10 minutes, and then stored at -20 °C. When all the samples were collected, they were tested against the indicator *L. lactis* (B1403).

2.11 Proteinase K test and heat treatment

This experiment was conducted to test whether the antimicrobial molecule is constituted from proteins. Simultaneously it would be checked if the samples were stable under higher temperatures. The purified antimicrobial compounds had been filtrated through a 0.2 µm syringe filter (Sarstedt) to prevent any bacterial growth. A buffer was then made to incubate the antimicrobial compounds with the proteinase K before testing it on an indicator. A TBS buffer of 50 mMol concentration with 5 mMol CaCl2 and 150 mMol NaCl was prepared, and was mixed 15 µl with 15 µl each of the purified antimicrobial. This ensured that the isopropanol in the antimicrobial fraction was low enough to not interfere with the function of the proteinase K (Tomar et al., 2009). To the test samples was added 3 µl of 20 mg/ml of proteinase K. Control samples were not mixed with the proteinase K. Garvicin KS was used as a control, and sample of Garvicin KS was mixed with proteinase K and another one without Proteinase K. Proteinase K would also be added on the indicator plate, as a negative control. The samples were incubated in PCR strips at 45°C

for 1 hour, except for the products of *E. faecalis* that was run for 1 hour at 30°C. To inactivate the proteinase K, the samples were heated at 95°C for 5 minutes.

Overnight cultures of indicators known to be inhibited by the respective antimicrobial substances were prepared. 100 µl of these were mixed in 5 ml soft agar each and applied in agar plates. Medium, temperature, soft agar and overnight broth were fitted for indicators known to be inhibited by the antimicrobials: *L. curvatus* (B2355) in MRS at 30°C for the *E. thailandicus* product, *L. lactis* (B1403) in MRS at 30°C for the *E. faecalis* product and *S. parauberis* (sample #451) in GM17 at 37°C for the *S. uberis* products. Finally, 3 µl of the antimicrobial samples that were subjected to the PCR heater and heat treated, were applied on their respective agar plates. The plates were then grown overnight at the same temperature as the indicators were, and inhibition zones were subsequently observed.

2.12 Protein purification

In order to characterise the bacteriocins discovered, a purification procedure in several steps was necessary.

2.12.1 Ammonium sulphate precipitation

Each round of purification was done with one liter of liquid nutrient medium given a 2% inoculum. For the *E. thailandicus* this was 24 hours in MRS broth at 30 °C, as these conditions showed greatest production of bacteriocin for this strain (Holivololona, 2018). For the *E. faecalis* the incubation was 24 hours at 30 °C. Lastly, the *S. dysgalactiae* was incubated for 24 hours at 37 °C. These times were chosen because of the results of the antimicrobial production over time in results section 3.14.

A separation of cells from the supernatant was achieved by centrifuging the 1 liter cell solutions at 10000 x g at 4°C for 35 minutes. The supernatant was transferred into glass bottles, and the pellet of cells was disposed of. In order to precipitate the proteins in the solution, it was treated with ammonium sulphate. This removes the water between the protein molecules, pressing them out of the liquid. In order to reach a high enough concentration, an online ammonium sulphate calculator was used.
http://www.encorbio.com/protocols/AM-SO4.htm
A total of 373 g og ammonium sulphate was added while continuously swirling the bottles at 4°C until dissolved. The solution was then left overnight at 4°C.

Next day the solution of ammonium sulphate was centrifuged at 12000 x g for 45 minutes. Separation of pellet and solution was followed, with the protein pellet being kept and later dissolved in 200 ml MilliQ water.

2.12.2 Cationic exchange chromatography

The first step of purification was made by utilising a cationic exchange HIPrep 16/10 SP-XL column (GE Healthcare Biosciences). In this technique cationic molecules are sequestered from non-cationic molecules. Bacteriocins are cationic, and so they will be separated. The cation exchange column achieves this by containing resins that function as acids bound to ions of a cationic nature. The binding captures certain molecules while allowing other non-cationic molecules to pass through. Subsequently the bound cations can be exchanged by a salt and then released into a liquid.

To equilibrate the column, a solution of distilled water added 1 M HCl adjusted to a pH of 4.0 and pumped through the cationic exchange column. While this processed, the protein solution from the centrifugation was adjusted to the same pH as the equilibration solution. For the NKR 5-3B the pH was 4.0, for the antimicrobial produced by *E. faecalis* the pH was 4.0, while the pH was 4.0 for the antimicrobial produced by *S. uberis*. The solution with the proteins in question was administered through the column, keeping the flow-through. Next, 100 ml of a phosphate buffer made by a combination of Na_2HPO_4 and NaH_2PO_4 (267 µl and 65 µl, respectively, in 100 ml distilled water) was applied to the column in order to remove impurities still present in the exchange column. The flow-through was kept. In order to eluate the proteins bound to the column, 100 ml of 1 M NaCl was applied through the column, followed by a higher concentration of 2 M NaCl to make sure that there were no more proteins attached to the column. Both the flow-throughs were kept along with the other samples taken during the process and stored at -20°C. The supernatant, flow-throughs, phosphate washes, and NaCl elutes were tested against a given indicator in a microtiter assay. For *E. thailandicus* this was *L. curvatus* (B2355), for *E. faecalis* it was Llactis B1403) and for *S. uberis* it was the sample #451, *S. parauberis*.

2.12.3 Reverse phase chromatography

The eluted protein solution from the cationic exchange chromatography was further purified by using an ÄKTA Purifier (Pharmacia Biotech) connected to a sample collector. In this technique, the polar mobile phase of the sample is passed through a non-polar and immobile phase. The molecules being transferred through bind to the immobile phase. Later, this allows for elution according to charge, with molecules being faster eluted the more polar they are. These are eluted by a gradual increase in the proportion of isopropanol used to elute the proteins.

Two solutions were prepared. One solution A was made with filtered water and 0.1% TFA, the other, called B, was pure isopropanol containing 0.1% TFA. Before the sample from the cationic exchange chromatography was sent through the column, it was washed and equilibrated with the water and TFA solution. After this, the sample from the cationic exchange purification was run through the column. To elute the proteins in separate fractions they were transported into separate glass tubes by an increasing degree of buffer solution B. Thusly they were eluted in order of increasing hydrophobicity, since this was 2-propanol with 0.01% TFA. The sample collector had glass tubes inserted, and these fractions were later tested in a microtiter assay against an indicator sensitive to the resulting proteins to identify the active fractions from the protein purification.

2.13 MALDI-TOF (Matrix Assisted Laser Desorption/Ionization-Time of Flight)

This technique was done to reveal the masses of the purified bacteriocins. The principle is that a UV laser is used to deliver energy into a sample that has been prepared with a crystallising matrix. A TOF mass analyser unit is also attached to the MALDI laser. It contains a drift region that allows the ionised protein samples to move down the region. Ion generation occurs within a defined time. The “time of flight” (TOF) for the ionised protein is proportional with the ratio of mass/charge, called m/z. This allows for determination of mass in the sample by use of a spectrometer (Rose et al., 1999). MALDI is especially useful for proteins. It creates singly charged ions for the most part and thus can include larger amounts of proteins in the sample (Croxatto et al., 2012). Masses of peptides can thus be visualized and used to compare with masses of bacteriocins already known

A HCCA (α-Cyano-4-hydroxycinnamic acid) matrix solution that absorbs UV light within the range that the laser uses. The matrix had been prepared according to manufacturer's direction beforehand. This matrix was mixed 1:1 with 0.5 µl sample each on a metal target plate (Bruker Daltonic) specific for MALDI procedures. In addition to the samples themselves, MALDI standard calibration peptides were also mixed with the HCCA matrix solution, both 0.5 µl each. The samples on the plate were then placed in a chamber in the MALDI device, under the guidance of Kirill Ovchinnikov at the LMG lab. The laser was then calibrated by analyzing the calibration peptides first, then analysis of the samples themselves ensued. Relevant peaks appearing were stored, and several uses of the laser to ionise samples were used to get a reliable result. The peaks occurring are detected and stored as data. Later this is used to compare with the results from the genomic sequencing.

3.0 Results

Considering that the project was in two parts, the results section was divided according to part one and part two for ease of interpretation. Part 1 follows the route of the purification and mutant generation of NKR 5-3B. Part 2 describes the screening, purification and characterisation of the bacteriocin producers identified from the raw milk samples.

3.1 Part 1

3.2 Ammonium sulphate precipitation and cationic exchange

Based on earlier work, the *E. thailandicus* strain was inoculated in MRS broth at 2% inoculum 30 °C for 24 hours, as any time after this would see the bacteriocin starting to degrade (Holivololona, 2018). The ammonium sulphate precipitation and cationic exchange chromatography was followed as described in section 2.12.1 and 2.12.2, respectively. The different phases from the purification is summarised in table 3.1. The antimicrobial activity is indicated as BU/ml, and the activity was determined by microtiter antimicrobial assay tested with *L. curvatus* (B2355) as indicator. The activity is increased in the purification process.

Table 3.1 *antimicrobial effect by step in the purification of NKR 5-3B. The steps are supernatant (SN), ammonium sulphate precipitation (AS), cationic exchange 1M NaCl eluate (CE) and reverse phase chromatography (RPC).*

Phase	Volume, ml	BU/ml	BU total	Yeld % total
SN	1000	40	40000	100
AS	400	160	64000	160
CE	200	320	64000	160
RPC	3	51200	156300	390

3.3 Reverse phase chromatography

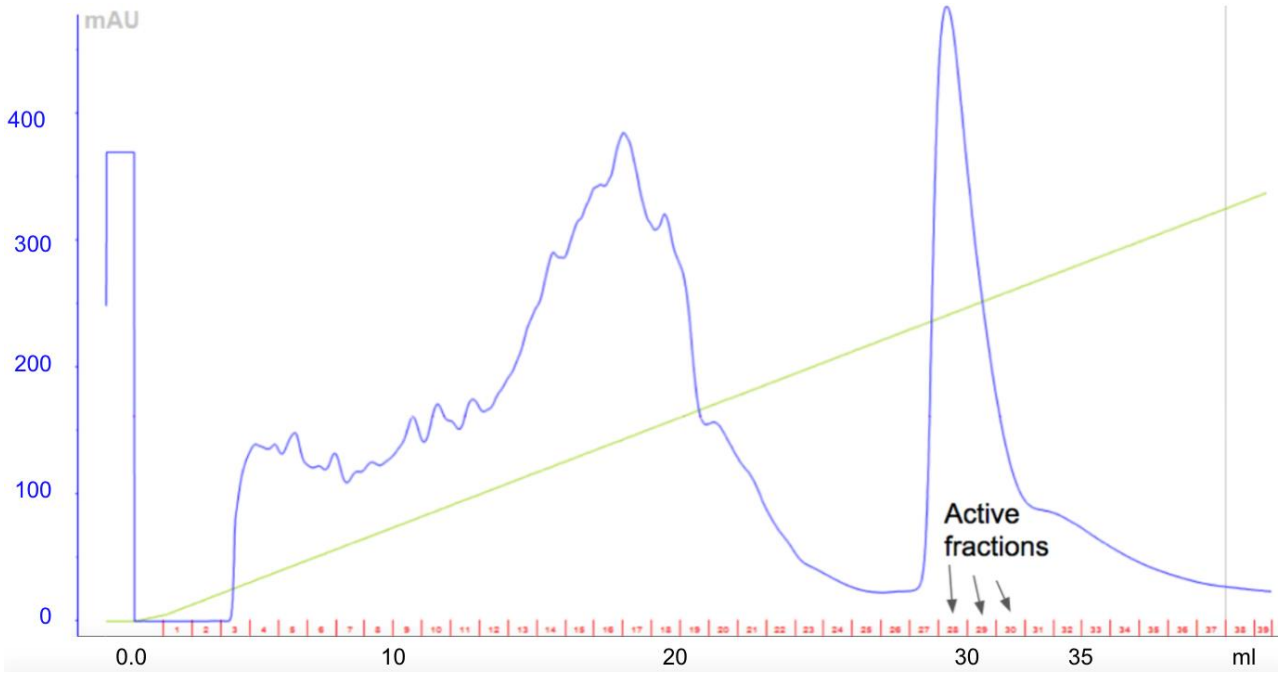


Figure 3.1 *ÄKTA reverse phase chromatography. Blue graph indicates absorbance at 280 nm, green line is percentage 2-propanol. The active fractions 28-30 were eluted at 48% 2-propanol.*

The proteins in the sample from the cationic exchange was applied to the ÄKTA reverse phase chromatography. A UV detector shows the absorbance at 280 nm. All the fractions

were tested against the same indicator employed for testing activity in the cationic exchange chromatography. As can be seen in figure 3.1, the most active fractions are #28-30, and it corresponds with the largest peak in the spectrum.

3.4 Mass spectroscopy

The active fractions 27-31 from the protein purification displaying the greatest antibacterial potential was subjected to MALDI-TOF analysis by Kirill Ovchinnikov at the LMG laboratory according to the procedure in section 2.13. The result is displayed in figure 3.

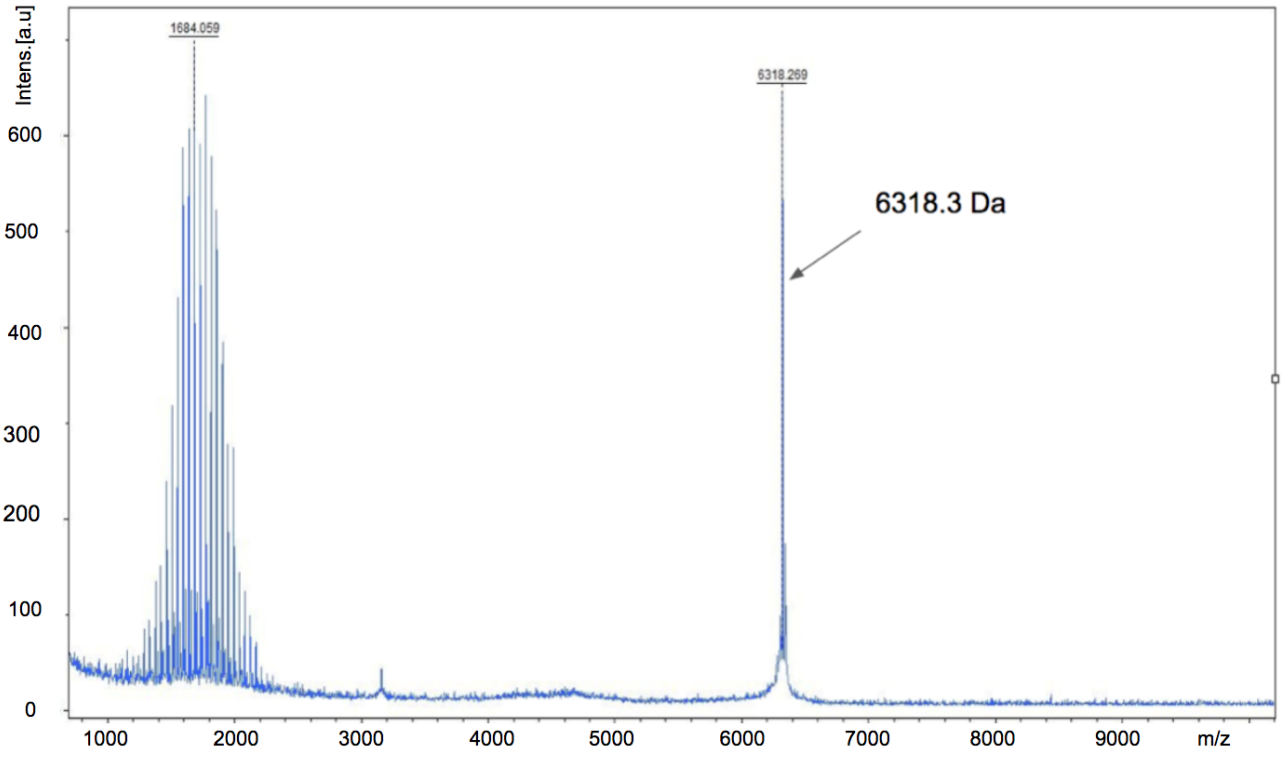


Figure 3.2 *MALDI TOF Mass spectra of NKR 5-3B from the purified antimicrobial substance.*

A search for the theoretical weight of NKR 5-3B indicates that its size is 6316.4 Da. In figure 3.2 it can be observed that there is a peak at 6318.3 Da. This is what corresponds to NKR 5-3B, or thaiocin 1. It is very likely that this is the active bacteriocin in the sample. There is a collection of smaller peaks at 1684 Da. These also occurred in previous work on this bacteriocin (Holivololona, 2018). It can be interpreted as detergents occurring in the

sample that have not been removed by the AKTA purifier during the reverse phase chromatography.

3.5 Proteinase K test

The NKR 5-3B was tested, and seems to be both temperature stable and resistant to proteinase. Since the identity of this bacteriocin is known, it is known that a circular bacteriocin such as this is resistant to proteinase K and very thermostable (Perez et al., 2015). Therefore this does not contradict that the bacteriocin is proteinaceous.

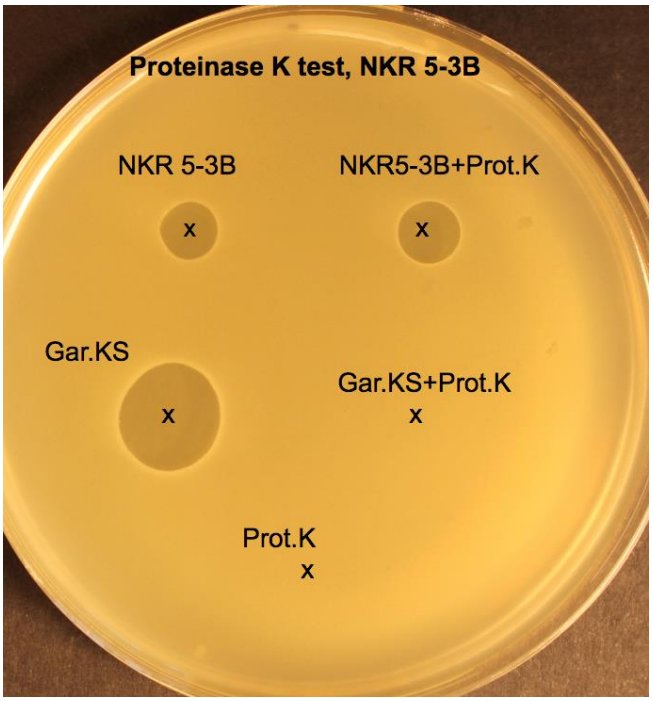


Figure 3.3 *Proteinase K test for NKR 5-3B vs. L. garvieae B3390 (upper right). Only temperature test is in upper left. The proteinase and sample were incubated for 1 hour before test against the indicators.*

3.6 Inhibition spectrum by spot-on-lawn assay

The antimicrobial spectrum of the purified bacteriocin was tested using the spot-on-lawn technique. A collection of 42 different strains were used as indicators to obtain data regarding the range of inhibition that the NKR 5-3B/ thaiocin displays. In addition, x10 and x100 dilutions were tested to qualitatively gain insight into the activity of the bacteriocin.

Nisin A and garvicin ML were supplied by Kirill Ovchinnikov at the LMG laboratory and applied side by side with NKR 5-3B. Nisin A is both broad spectrum and potent, while garvicin ML is a circular bacteriocin, like NKR 5-3B (Borrero et al., 2011; Shin et al., 2016). They were therefore deemed appropriate for comparison with NKR 5-3B.

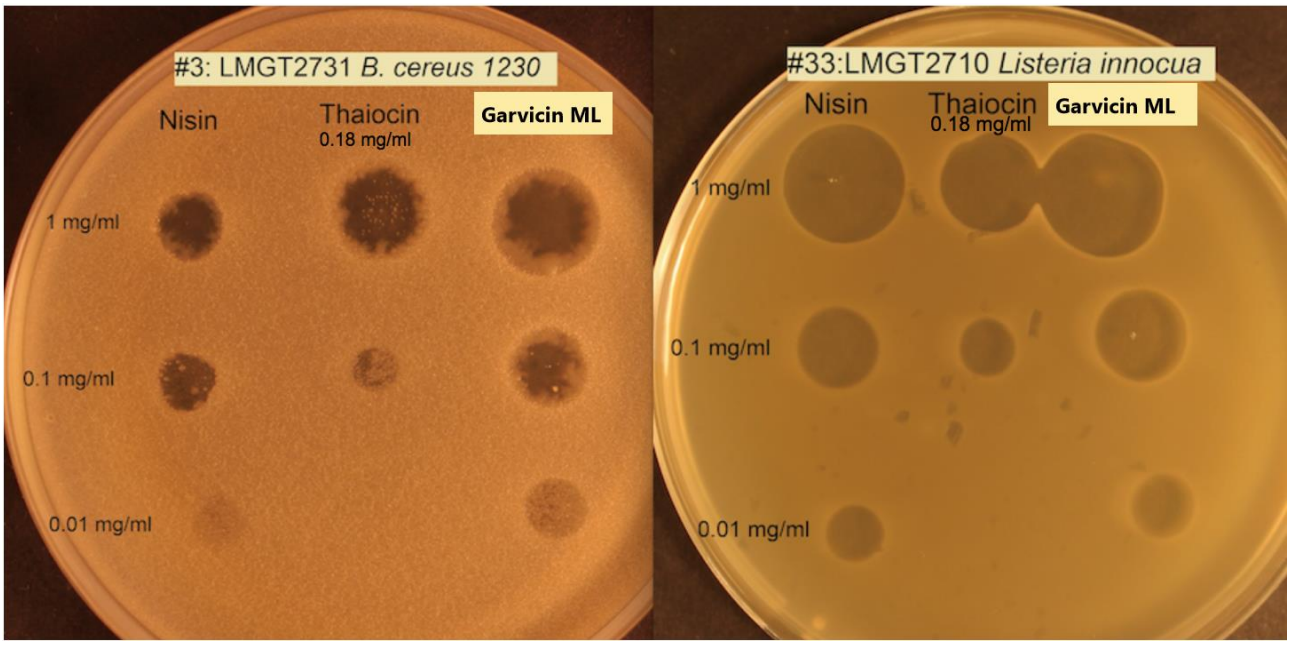


Figure 3.4 *Example of results from the inhibition spot-on-lawn assay for NKR 5-3B.*

The concentration for NKR 5-3B under the inhibition spectrum was 0.18 mg/ml, as it was after protein purification. The concentration was obtained by measuring the purified NKR 5-3B on a NanoDrop ND-1000 (NanoDrop Technologies). The concentrations for nisin A and garvicin ML were both 1.0 mg/ml. There were also some indicators that produced observable colonies in the inhibition zones, as can be seen for *B. cereus* (B2731) in figure 3.4.

In table 3.2 the inhibition spectrum of NKR 5-3B is shown. The antimicrobial effect is broad as is expected for circular class Ib bacteriocins (Perez et al., 2018). Only *E. coli* and *S. aureus* were unaffected by NKR 5-3B.

Table 3.2 *Inhibition spectrum of NKR 5-3B tested against 42 indicators. Points given are 0 (no inhibition zone) to 3 (three clear inhibition zones).*

Indicator	**Bacteriocin zones of inhibition (average)**		
	NKR 5-3B	**Nisin A**	**Garvicin ML**
L. monocytogenes (n=5)	1.5	2	2.5
L. innocua (n=2)	1.5	2.5	2.5
L. Ivanovii (n=1)	2	2	3
B. cereus (n=4)	2	2	3
S. aureus (n=3)	0	2	2
C. divergens (n=1)	1	2	2
E. faecalis (n=5)	1	2	2
E. faecium (n=4)	1	1.5	2
E. avium (n=1)	2	3	3
L. plantarum (n=3)	1	2	3
L. sakei (n=2)	1.5	2.5	3
L. curvatus (n=2)	1	3	3
L. lactis (n=2)	1	2.5	3
L. garvieae (n=1)	2	3	3
S. thermophilus (n=1)	2	2	2
S. uberis (n=1)	1	3	3
S. dysgalactiae (n=1)	1	2	2
L. gelidium (n=1)	2	1	3
E. coli (n=2)	0	0	0

3.7 Generation of mutants

As explained in section 3.2, inhibition zones did in some cases allow for cells to grow within them. These were picked with sterile toothpicks and further spread on new agar plates to generate single colonies. After growth in BHI broth, they were then tested against the NKR 5-3B bacteriocin to see their potential resistance against it. Any colonies appearing were then tested again to see if they had a stable resistance against the bacteriocin. The outcomes are shown in figures 3.5 - 3.7.

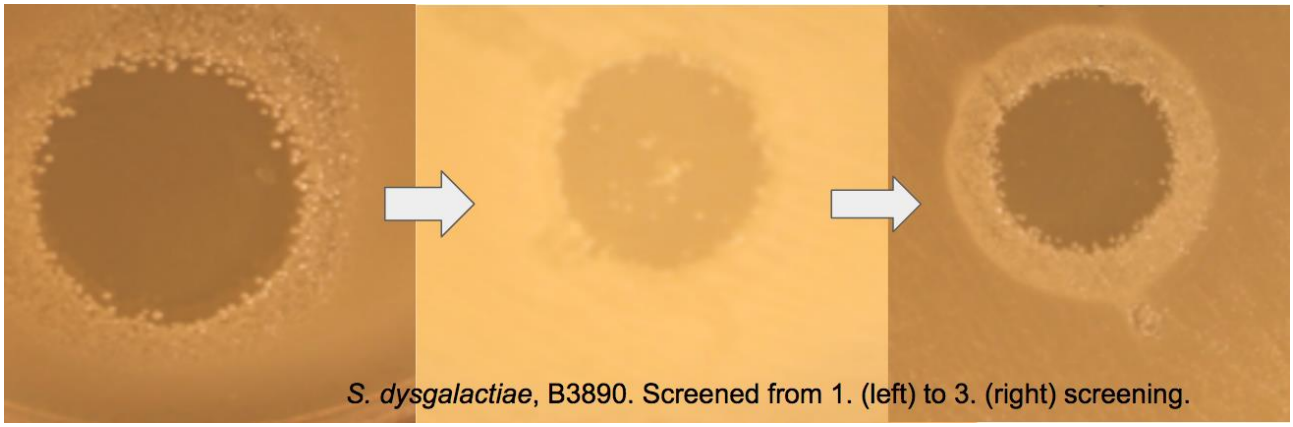


Figure 3.5 *Attempts to generate and isolate mutants for S. dysgalactiae (B3890).*

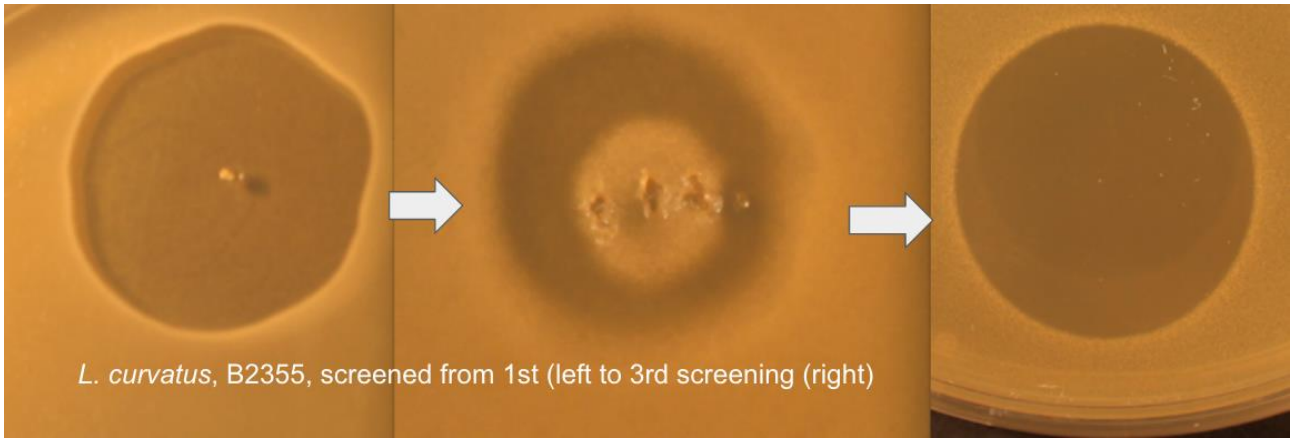


Figure 3.6 *Attempts to generate and isolate mutants for L. curvatus (B2355).*

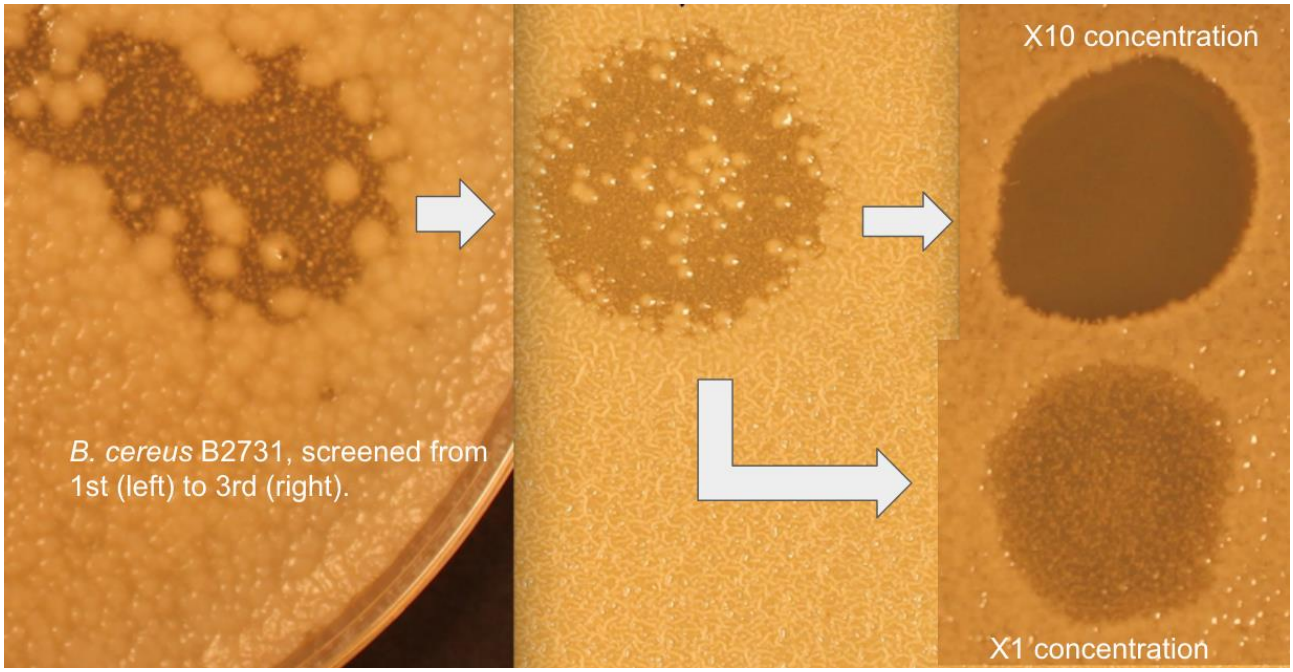


Figure 3.7 *Attempts to generate and isolate mutants for B. cereus (B2731).*

The three strains that showed further growth in the second screening were *S. dysgalactiae (*B 3890), *L. curvatus* (B2355), and *B. cereus* (B2731). But the result was still an inhibition zone, even when repeated. In order to completely exclude mistakes as a source of this outcome, the colonies from the second screening were also picked with sterile toothpicks, spread on new BHI plates to generate single colonies. Then they were inoculated in 5 ml BHI broth and tested again to see if they were resistant to the NKR 5-3B. For the *B. cereus* (B2731) the purified bacteriocin was also concentrated x10 by the aid of a Speedvac Vacuum concentrator (Thermo Fischer) at 30 °C. In figure 3.7 it can be seen that there are colonies at the concentration used when attempting to generate mutants. But when a 10x concentration of the bacteriocin is applied, there is just a clean inhibition zone. There are resistant bacteria that appear in some inhibition zones for a few indicators, but the isolates from the zones do not show an inheritable, stable resistance that they all display when tested again. Thus, it was not possible to generate mutants in the timeframe of the project. This impedes further progress of this part of the project, as there are no resistant mutants to compare with the wild type.

3.8 Part 2

3.9 Screening for bacteria

As described in section 2.3, the samples were isolated from the indicator by a layer of soft agar and allowed to incubate at 30°C under aerobic conditions overnight. While there were no results for the combined *Enterococcus faecalis* (B3330) and *Staphylococcus aureus* (B3325) mixed together, the three separate indicators *Streptococcus dysgalactiae* (B3890), *Staphylococcus aureus* (B3023), and *Enterococcus faecalis* (B2333) led to some samples creating inhibition zones. These are summarised in table 3.3, along with the indicator used, and the name they were given. A few samples produced a prodigious amount of inhibition zones, as can be seen in figure 3.8 far left. These were picked with sterile toothpicks, and put in 15% glycerol and stored at -80 °C until further use.

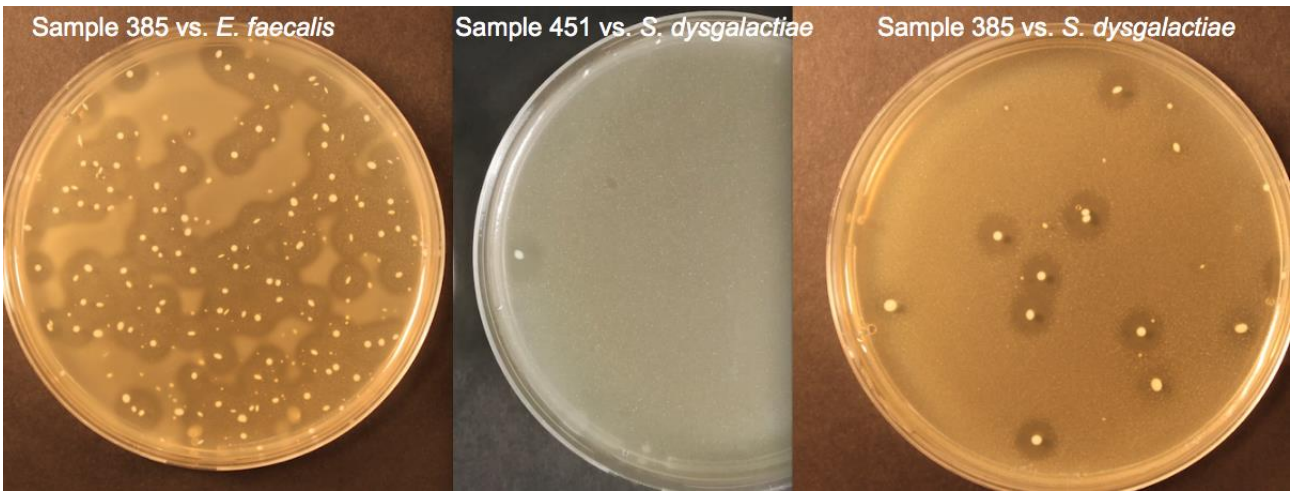


Figure 3.8 *Visual results of some screening samples with inhibition zones*

Table 3.3 *Samples obtained from the screening process, indicator used, and their names*

Sample	Concentration	Indicator	Sample name(s)
#385	0.1	*S. dysgalactiae,* B3890	#1-10
#385	0.01	*E. faecalis,* B2333	#11
#385	1	*E. faecalis,* B2333	#12-36

#385	0.1	*E. faecalis,* B2333	#37-45
#269	1	*S. dysgalactiae,* B3890	#269
#451	1	*S. dysgalactiae,* B3890	#451

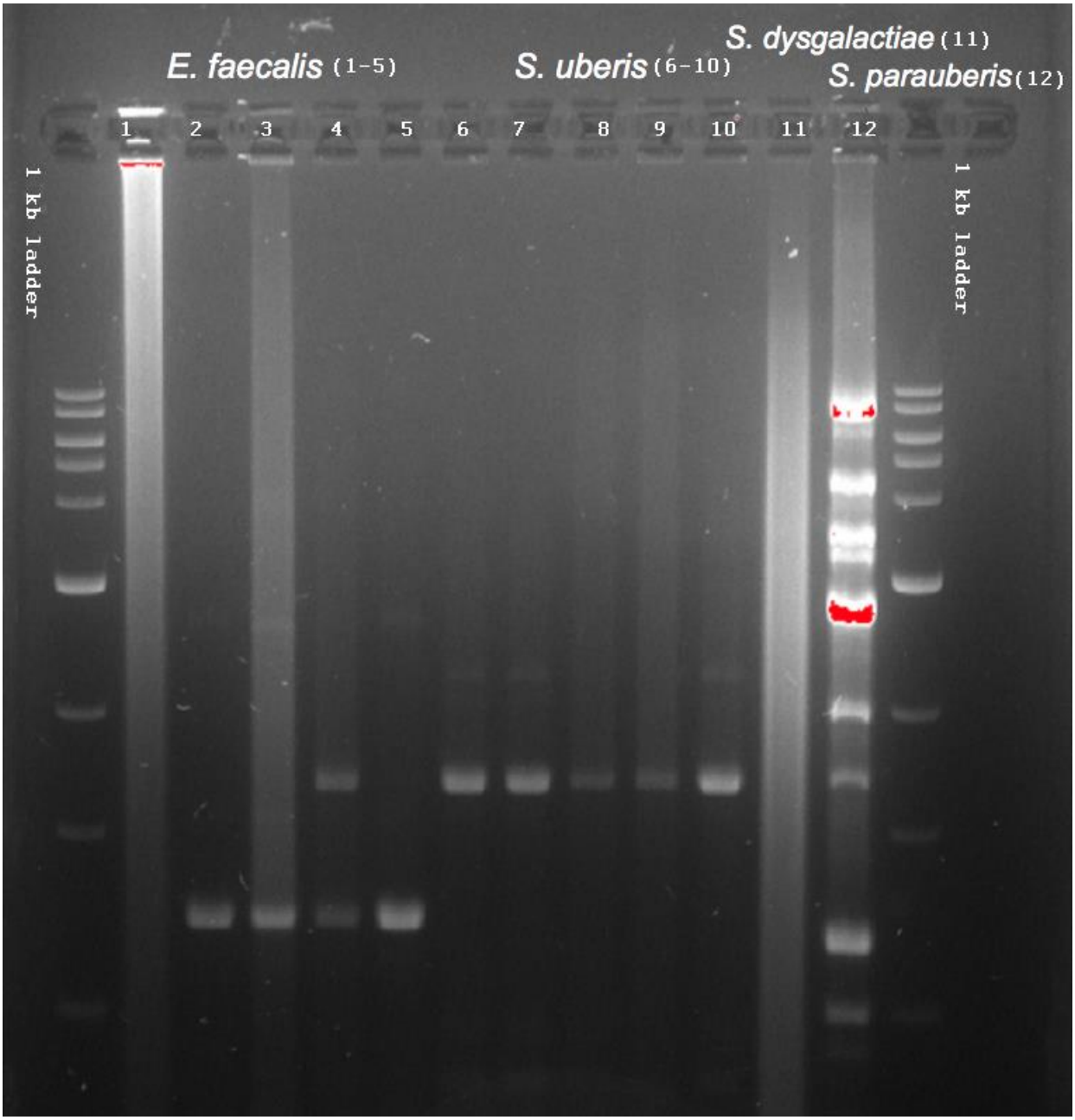
3.10 16S rRNA PCR (polymerase chain reaction)

The results of the 16S rRNA PCR analysis were used by the Bioedit sequence alignment editor program to assess the accuracy of DNA information, and sampled information from this was used to perform a BLASTn search. The results from this would generate hits based on the information. In the samples, five are *S. uberis*, five are *E. faecalis*, and there is one *S. dysgalactiae* and *S. parauberis* each among the samples.

Table 3.4 *Samples' identity based on the 16S rRNA analysis. All the results had 96% identity or higher.*

Sample number	**Species**
2	*Streptococcus uberis*
5	*Streptococcus uberis*
8	*Streptococcus uberis*
11	*Enterococcus faecalis*
13	*Streptococcus uberis*
26	*Enterococcus faecalis*
30	*Enterococcus faecalis*
37	*Streptococcus uberis*
39	*Enterococcus faecalis*
43	*Enterococcus faecalis*
269	*Streptococcus dysgalactiae*
451	*Streptococcus parauberis*

3.11 ERIC (Enterobacterial Repetitive Intergenic Consensus)-PCR



Picture 3.9 *ERIC PCR for strains chosen. 1 kb ladders are chosen for the first and last well.*

The ERIC PCR reveals specific bands that would differ with variations in the DNA. The gel in figure 3.9 shows that well 1-5, which is *E. faecalis*, has bands that seem for be in the same positions, with the exception of the smear in well 1. This indicates that these strains are similar. Well 6-10, containing *S. uberis*, also had bands in the same positions on the

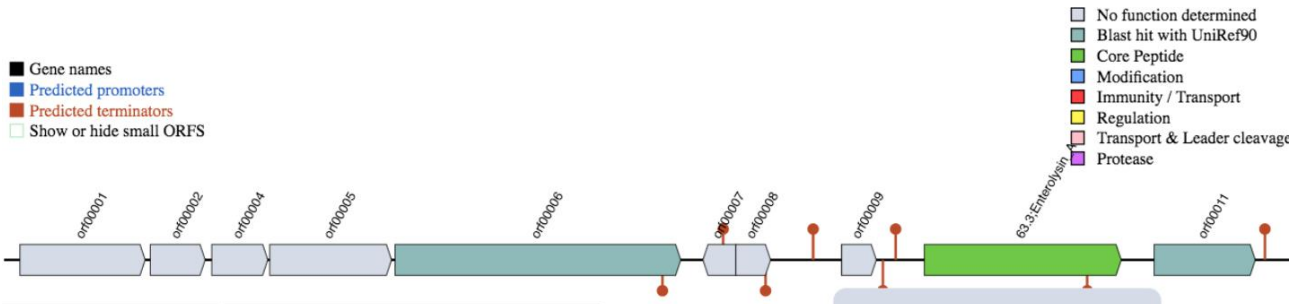
agar gel. This also gave reason to conclude that they were identical strains of *S. uberis*. Well 11 and 12, being *S. dysgalactiae* and *S. parauberis* respectively, were unique. These were added to show that they would be different than the other strains.

3.12 Genomic sequencing analysis

Based on the results from the 16S rRNA analysis, two each of *E. faecalis (#30 and #43)* and *S. uberis* (#8 and #13) were chosen, along with the *S. dysgalactiae (#269)* and *S. parauberis (#451)*, for whole genome sequencing. This was done by Davide Porcellato at the KMB department of Dairy Technology and Food Quality on a Miseq platform (Illumina). Data generated from the genomic sequencing were then annotated by RAST (Rapid Annotation using Sub-system Technology) and converted to FASTA files with the help of Thomas Oftedal at the LMG laboratory.

Identification of bacteriocin genes was done by uploading the FASTA files on the bacteriocin mining tool BAGEL (BActeriocin GEnome mining tooL) which contains catalogues of potential bacteriocins already discovered. A BLASTp (Basic Local Alignment Search Tool, Proteins) was performed as well to see how the query sequences matched. BAGEL site identifications would be identified further by BACTIBASE, a site containing bacteriocins identified and characterised.

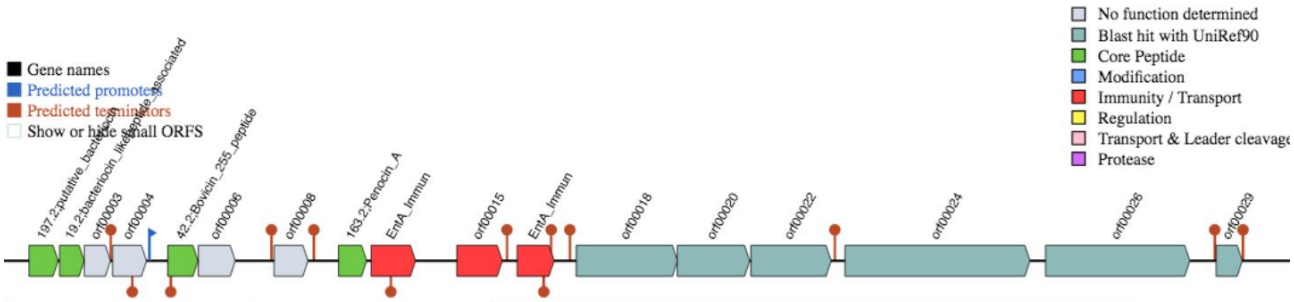
Only the *E. faecalis* #43 and the *S. uberis* #13 had been chosen to be further characterised, and so these are the relevant samples. Sample #13 did not produce any results on the BAGEL site. Based on the ERIC-PRC results in picture 3.9, the evaluation was that the *S. uberis* strains did not display any genetic diversity. Therefore, sample #8, also containing *S. uberis* that are genetically homogenous to the sample #13, was uploaded on BAGEL instead. Any findings would be deemed as representable for the further characteristics of all the identified *S. uberis*, including sample #13.



Gene name	Function/ Blast hit
ORF 00006	Staphopain A OS
63.3; Enterolysin_A	Enterolysin A
ORF 00011	Uncharacterised protein YvpB

Figure 3.10 *BAGEL4 results from sample #43 E. faecalis bacteriocin gene clusters and names of putative genes with BLAST hits.*

The generated BLAST hit in figure 3.10 for the putative enterolysin A shows a 100% identity match. This indicates that the bacteriocin produced by *E. faecalis* in sample #43 is Enterolysin A. As this bacteriocin is characterised and known, it was decided that further characterisation was not necessary, and sample #13 from *S. uberis* would be the primary candidate for purification and characterisation further in the process.



Gene name	Function/ BLAST hit
197.2; putative bacteriocin	Bacteriocin IIc putative bacteriocin
19.2; bacteriocin-like peptide associated	Bacteriocin IIc bacteriocin-like peptide
42.2; Bovicin 255 peptide	Bovicin 255 peptide
163.2; Penocin A	Penocin A antimicrobial
EntA Immun	Immunity protein
ORF 00015	Bacteriocin self-immunity protein
EntA Immun	Enterocin CRL 35 immunity protein
ORF 00018	Formamidopyrimidine-DNA glycosylate
ORF 00020	Dephospho-CoA kinase
ORF 00022	Uncharacterised protein HI 0522
ORF 00024	Uncharacterised protein YjgR
ORF 00026	Multidrug resistance efflux pump PmrA
ORF 00029	Protein export membrane protein

197.2;putative_bacteriocin
Function: ComC; Bacteriocin_IIc; 263.1;Gassericin197.2;putative_bacteriocin
Blast result UniRef90: NA; Bacteriocin class II with double-glycine leader peptide; Evalue=2e-15 match=48.53%;Evalue=2e-26 match=80.60%

163.2;Penocin_A
Function: Bacteriocin_II; Antimicrobial17; Bacteriocin_IIc; 163.2;Penocin_A
Blast result UniRef90: Class II bacteriocin; Alpha/beta enterocin family; Bacteriocin class II with double-glycine leader peptide; Evalue=2e-13 match=40.62%;

42.2;Bovicin_255_peptide
Function: ComC; Bacteriocin_IIc; 42.2;Bovicin_255_peptide
Blast result UniRef90: NA; Bacteriocin class II with double-glycine leader peptide; Evalue=2e-32 match=65.79%;

19.2;bacteriocin_likepeptide_associated
Function: Bacteriocin_IIc; 19.2;bacteriocin_likepeptide_associated
Blast result UniRef90: Bacteriocin class II with double-glycine leader peptide; Evalue=6e-18 match=61.67%;

Figure 3.11 *BAGEL4 results from sample #13 S. uberis bacteriocin gene clusters and names of putative genes with BLAST hits.*

From figure 3.11 one can observe that there are four different candidates for possible bacteriocins. The connected BLAST in the same figure reveals that they have all less than 100% identity. Most probable is the BAGEL reference “putative bacteriocin” with 80.6% identity.

3.13 Proteinase K test

Bacteriocins are proteinaceous, and a proteinase K test would specify further the identity of the substance secreted by the respective producers. The procedure is described in section 2.11. If there was an absence of inhibition zone where the combined proteinase K and antimicrobial had been applied, it would indicate that it was proteinaceous. Garvicin KS was used to check the function of the proteinase, and it this was used alone to see that heat treatment inactivates proteinase K.

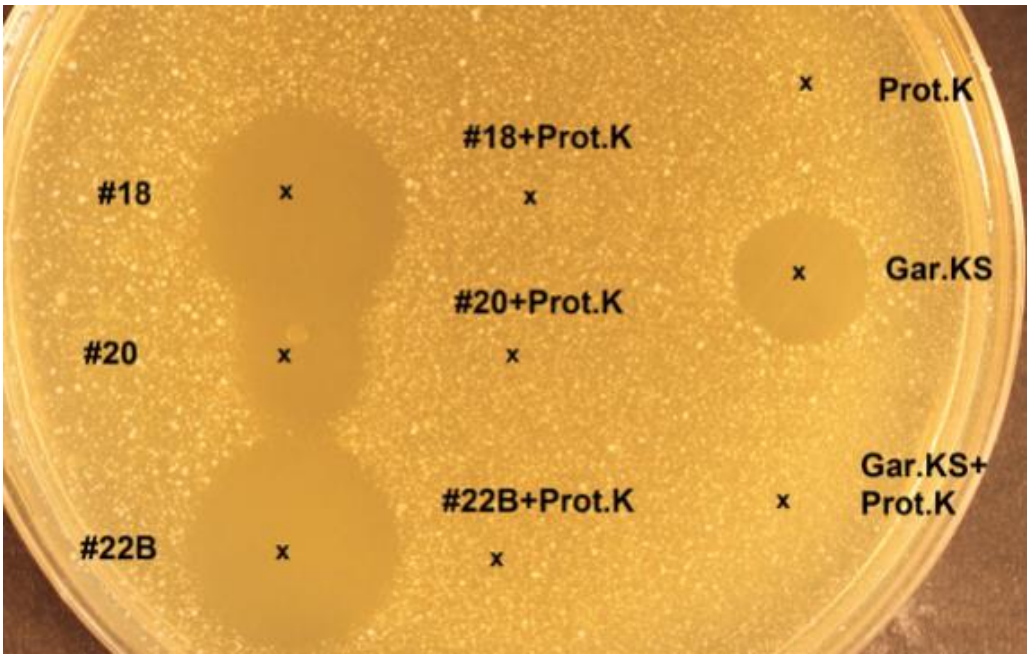


Figure 3.12 *Proteinase K test for fractions of purified protein by S. uberis #13 vs. #451 S. parauberis (left). The proteinase and sample were incubated for 1 hour before test against the indicators.*

There were several fractions produced from the purified antimicrobial by reverse phase chromatography of sample #13. Fractions #18, #20 and #22B were heat stable (left side on plate). These were inactivated by the proteinase (middle column), this indicates they are proteinaceous. Fraction #18 and #20 was from the first round of purification, fraction #22B was from the second round.
Sample #43 produced by *E. faecalis* was also subjected to proteinase K test, but was heat-labile and inactivated. It thus did not produce any inhibition zones either with or without proteinase K.

3.14 Antimicrobial production over time

To get as much as possible from the protein purification in section 2.12, both the *E. faecalis* from sample #43 and *S. uberis* from sample #13 were tested over 3 time intervals in different medium broths: GM17, BHI MRS and TH (only *S. uberis*). An aliquot of 1 ml was taken for each medium and time point. After filtration and storing at -20°C, they were all tested in microtiter assay described in section 2.9 against *L. lactis* (B1403).

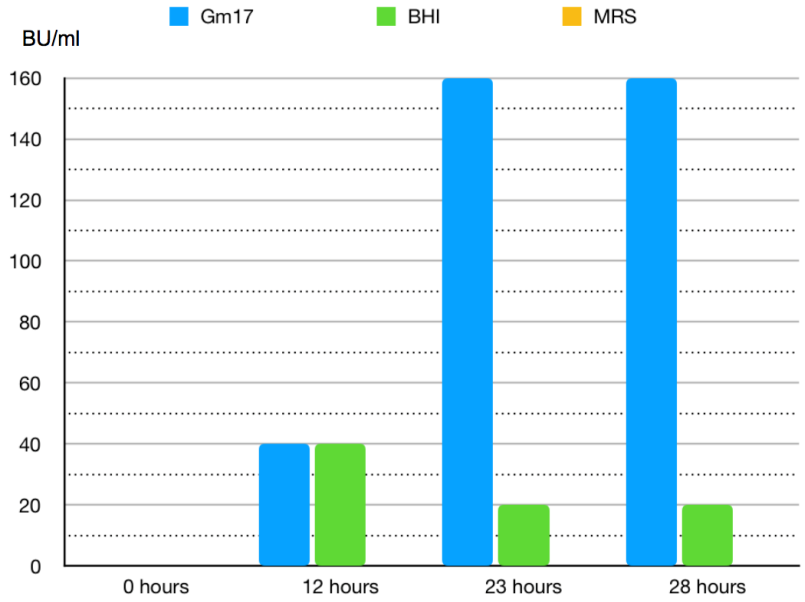


Figure 3.13 *Production of antimicrobial compounds over time, depending on growth medium for E. faecalis, sample #43. The indicator used was L. lactis B1403.*

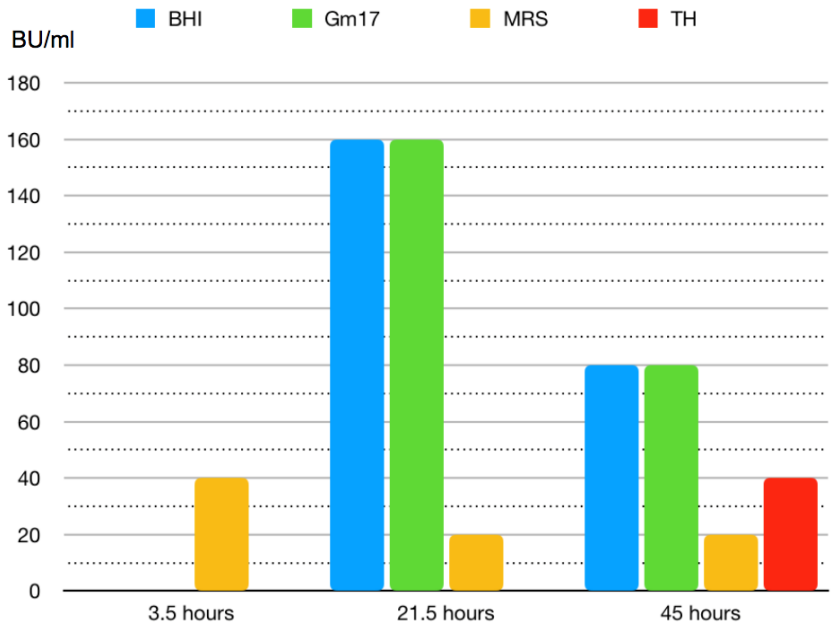


Figure 3.14 *Antimicrobial production in different growth medium for S. uberis, sample 13. This was tested against L. lactis B1403.*

It became evident that the isolate from sample #43 *E. faecalis* produced antimicrobials decidedly best in GM17. MRS resulted in no bacteriocin produced for this strain. Up to 28 hours there was no reduction in antimicrobial activity with GM17.
S. uberis did produce some interesting and surprising results, as it had very poor production of antimicrobials in the TH broth. However, both BHI and GM17 produced the best antimicrobial concentration. Also, after 21.5 hours there is a reduction or degradation of active antimicrobials in the BHI and GM17 medium.

3.15 Cationic exchange chromatography

Based on the results in section 3.14, the *E. faecalis* was inoculated in GM17 at 2% inoculum at 30 °C for ca. 22 hours. *S. uberis* was inoculated at 2% inoculum in BHI medium for 37 °C, also for 22 hours. After this the procedures in section 2.12.1 and 2.12.2 was undertaken for the ammonium sulphate precipitation and cationic exchange chromatography. Table 3.5 shows the activity as BU/ml from the different phases during the process. The activity was determined by microtiter antimicrobial assay tested with the sample #451, *S. parauberis*.as indicator.

Table 3.5 *Antimicrobial effect by phase for sample #13, by S. uberis. The steps are supernatant (SN), ammonium sulphate precipitation (AS), cationic exchange 1M NaCl eluate (CE) and reverse phase chromatography (RPC).*

Phase	Volume, ml	BU/ml	BU total	Yeld % total
SN	1000	20	20000	100
AS	150	80	12000	60
CE	100	80	8000	40
RPC	3	102400	307200	154

The test for *E. faecalis* in sample #43 failed, and before this could be repeated, the Covid-19 pandemic of 2020 made access to the university impossible.

3.16 reverse phase chromatography

The eluate from 1M NaCl obtained from cationic exchange chromatography were used on the AKTA RPC column. Both sample #13 and #43 were chosen for this procedure. The proteins in the sample was registered by a UV detector which shows the absorbance at 280 nm. Figure 3.14 and 3.15 shows the chromatography spectra for the antimicrobials from *E. faecalis* and *S. uberis*, respectively. The fractions obtained were all tested for activity against the same indicators as were used for the phases in cationic exchange chromatography, section 3.15.

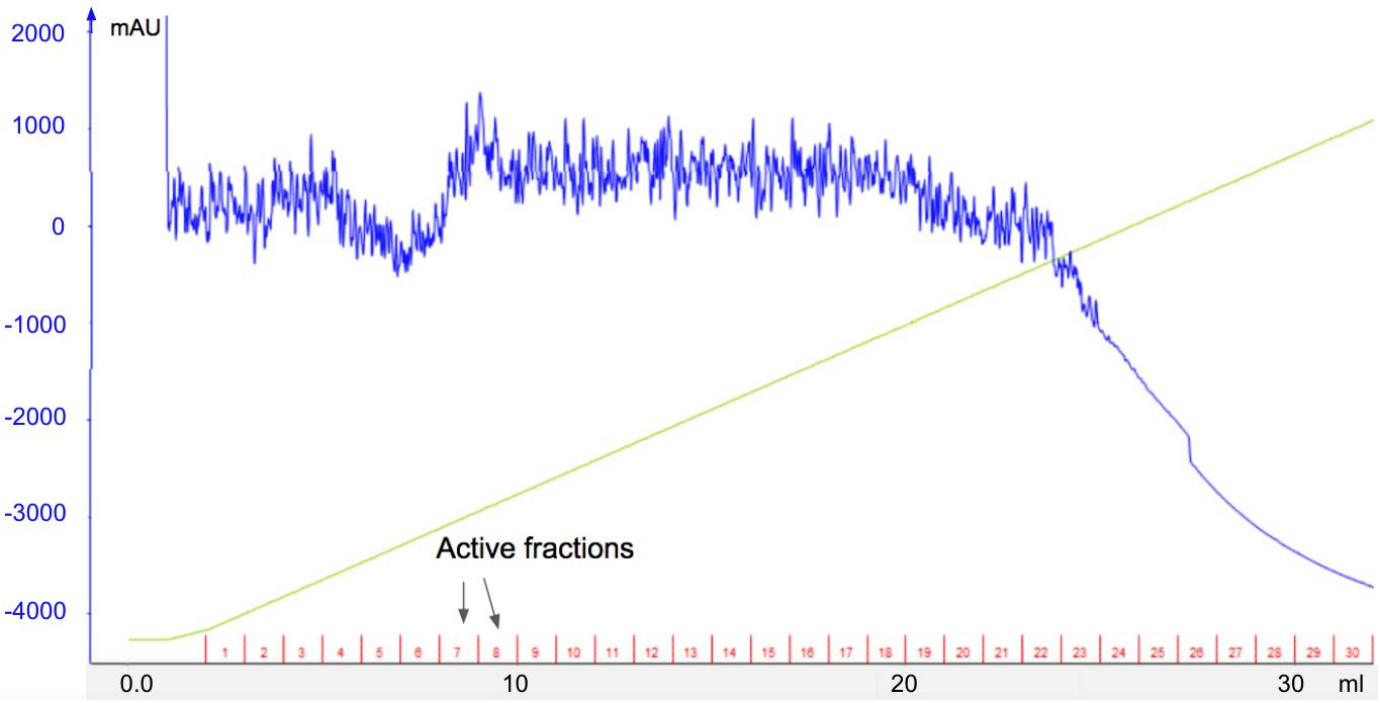


Figure 3.14 *ÄKTA reverse phase chromatography. Blue graph indicates absorbance at 280 nm, green line is percentage 2-propanol. The active fractions 7-8 were eluted at 14% 2-propanol.*

The spectrum for sample #43, *E. faecalis*, was not clear to interpret. There seemed to be a presence of proteins over the whole spectrum. There is a small peak were the active fractions are, at fractions 7 and 8.

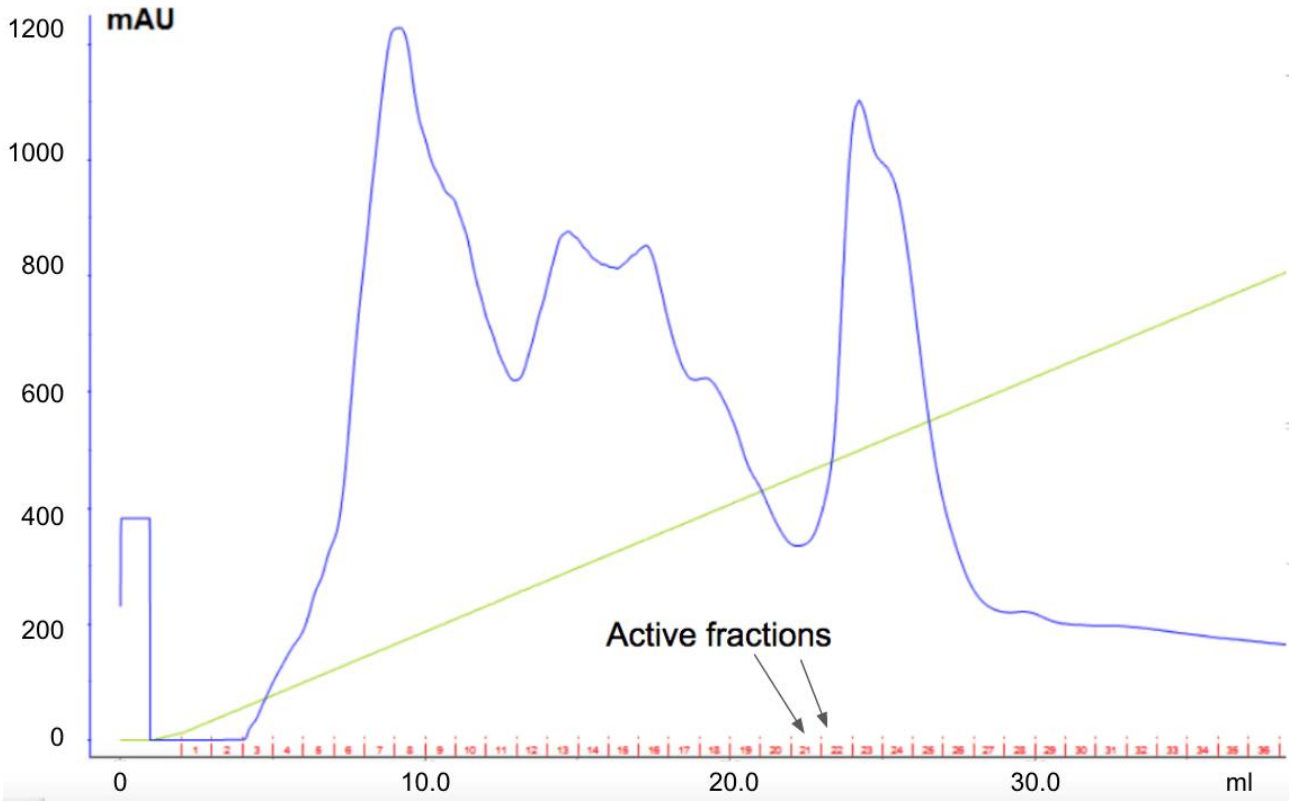


Figure 3.15 *ÄKTA reverse phase chromatography. Blue graph indicates absorbance at 280 nm, green line is percentage 2-propanol. The active fractions 21-23 were eluted at 29% 2-propanol.*

For the reverse phase chromatography used on the *S. uberis* eluate from the cationic exchange chromatography, the spectrum had more contrasts, but it still did not correspond to active fractions. In this case those were fractions 21 to 23. This was tested with the same indicator used to test for the cation exchange chromatography.

3.17 Mass spectroscopy

Since enterolysin A has been characterised, it was decided that sample #13 from the *S. uberis* producer would be chosen for mass spectrometry. The fractions that had the most antimicrobial activity (fraction #21 and #22) from the reverse phase chromatography were applied on the target metal plate for MALDI TOF and subjected to analysis.

The most probable bacteriocin from the BAGEL4 analysis was the “putative bacteriocin” with a 80.6% identity. The amino acid sequence obtained from BAGEL4 was used with the

EXPASY site to determine the theoretical mass, in order to compare this with the findings from the MALDI TOF results.

*T**able 3.6** Determining the mass putative bacteriocin from BAGEL4.*

Leader peptide	VDILMNSNIEFDSIDTELLEKVIGG
Mature peptide	KNNWQANVSGILAAGAAGAAIGAPVCGLACGYIGAKTAITLWAGVTGATGGF
Monoisotopic mass	4860.48 Da

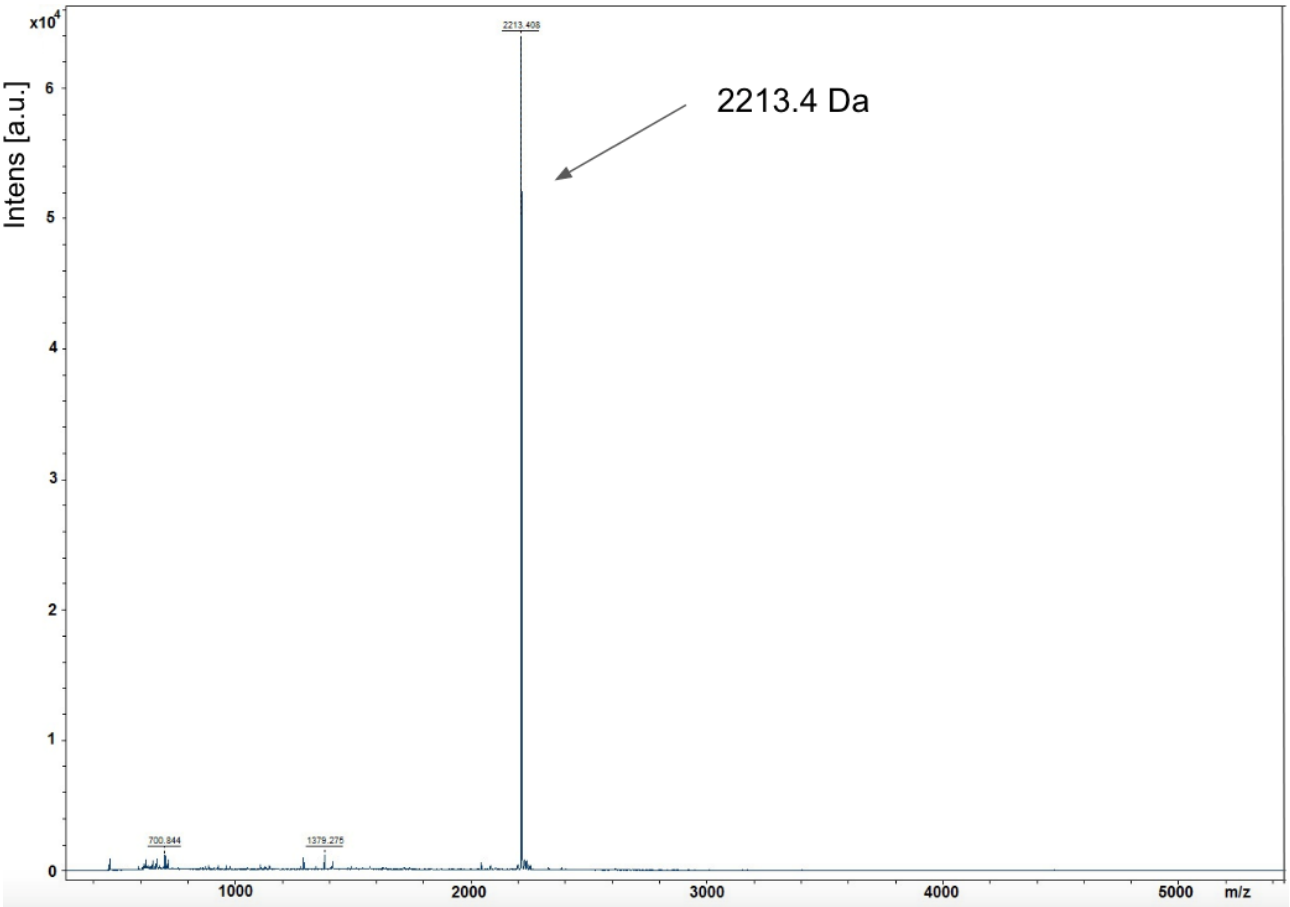


***Figure 3.16** Mass spectra of fraction #21 from the purified antimicrobial substance by S. uberis.*

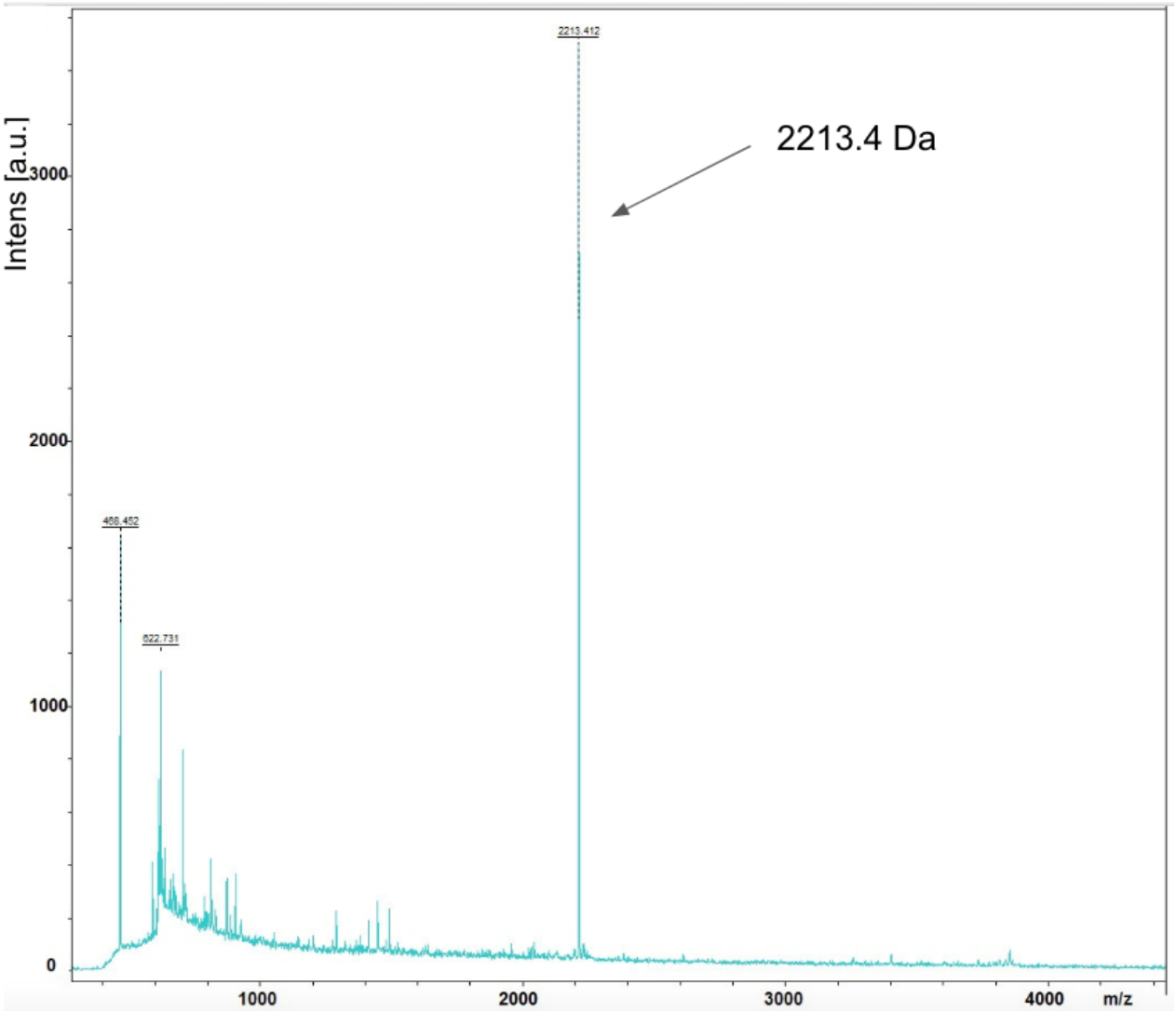


Figure 3.17 *Mass spectra of fraction #22 from the purified antimicrobial substance by S. uberis.*

Both of the purified fractions from the reverse phase chromatography has the same peak with similar mass of 2213.4 Daltons. And it does not correspond to any of the masses from the other bacteriocins from the BAGEL4 results. BACTIBASE was also browsed according to bacteriocin mass, but none corresponded to the peak from the mass spectroscopy. It could be theorised that the peak could be an ionised version of the bacteriocin, with peak double that of the mass. But no such peak could be located. The findings are thus that

there seems to be a peak in the samples at 2213.4 Dalton, which does not correspond to any bacteriocins currently known.

3.18 Inhibition spectrum

In order to characterise the antimicrobial from the *S. uberis* strain from sample #13 further, a spot-on-lawn assay was performed with 18 indicators to get an indication of the range of inhibitory action the peptide displayed. Nisin A was used as a comparison with 1 mg/ml concentration. Nanodrop measurement showed that the concentration of the purified antimicrobial from sample #13 was 0.289 mg/ml. There were also x10 dilutions for both the nisin A and the purified antimicrobial from sample #13. An unfiltered supernatant from the producer was also tested.

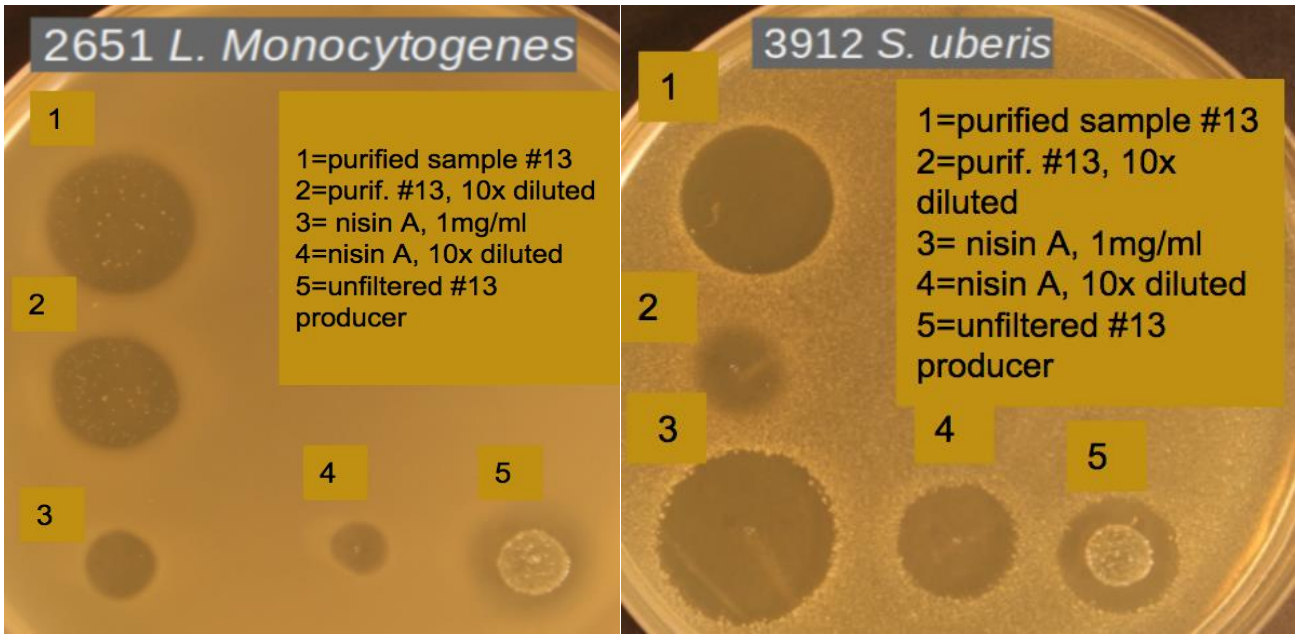


Figure 3.18 *Spot on lawn assay results for two samples.*

Table 3.7 *Results of the antimicrobial spectrum for purified antimicrobial from sample #13 against 18 indicators. Points of 2 is two clear inhibition zones (maximum), while 0 is no inhibition.*

Indicator	**Bacteriocin zones of inhibition**		
	RPC active fraction from sample #13	**Nisin**	***S. uberis* producer**
B. cereus (n=2)	0	1.5	0
E. faecalis (n=2)	0.5	2	0
E. faecium (n=2)	1.5	2	0
L. curvatus (n=1)	1	2	1
L. garvieae (n=1)	1	2	0
L. monocytogenes (n=2)	2	2	1
S. aureus (n=2)	0	1.5	0
S. dysgalactiae (n=1)	0.5	2	1
S. uberis (n=4)	2	2	0.5
E. coli (n=1)	0	0	0

Based on the results in table 3.7, it is apparent that the antimicrobial is very effective agains *L. monocytogenes*, *S. uberis* and *E. faecium.* It does not affect *E. coli, S. aureus* and *B. cereus.*

3.19 Mutual inhibition

In order to test the variation in antimicrobial effect between the strains of bacteria in the samples in part 2, a mutual inhibition test was performed, in which each bacteria from the screening was tested as the indicator against the others chosen from the screening. *L. lactis* B1403 was also added. The *S. dysgalactiae* and *S. parauberis* was in the top row, 5 *E. faecalis* middle row, and *S. uberis* at the bottom row.

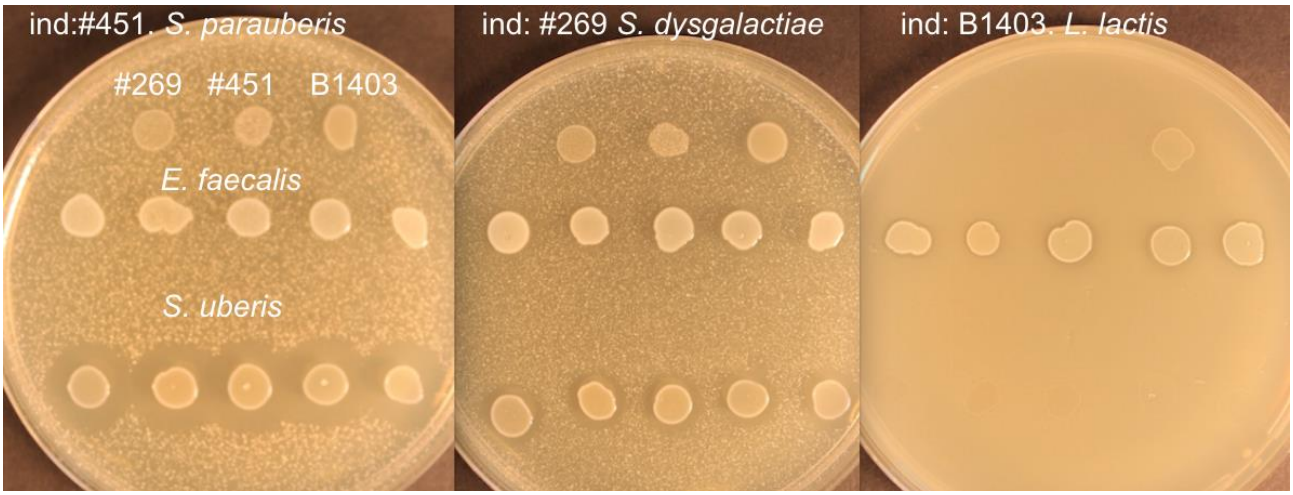


Figure 3.19 *The active results for the mutual inhibition test. The arrangement of spots is similar for all three plates.*

As can be seen in figure 3.19, there are some mutual inhibitory effects, and only the agar plates that displayed this was included here. In the left and middle picture, the *S. uberis* clearly inhibits the *S. parauberis* and *S. dysgalactiae*. At this point, it was decided to use #451 *S. parauberis* as an indicator to the *uberis* strains forward. At the far right picture, it can be observed that the *E. faecalis* inhibits the *L. lactis* indicator. The characteristic all the rows have in common, is that the related strains all inhibit the indicator. They display similar inhibitory effect. This further indicates that they are genetically similar.

4.0 Discussion and conclusions

4.1 Part 1

This part of the project set out to characterise receptors in the target bacterial cells subjected to the bacteriocin NKR 5-3B, also called thaiocin 1. The producer had already been isolated and characterised. The purpose was to test the inhibition spectrum and investigate the mechanisms by which the bacteriocin impacted its bactericidal effect on target cells. Purification of the bacteriocin was commenced early in the thesis. Both cationic exchange methods and reverse phase chromatography was used to end up with 3 ml of a purified bacteriocin with a concentration of 0.18 mg/ml from the reverse phase

chromatography process. The mass observed during the MALDI TOF corresponded to the theoretical mass of NKR 5-3B.

Test of the effect proteinase K and high temperature had, revealed that the bacteriocin is resistant both to both types of treatment. This is in accordance with circular bacteriocins being resistant to extremes of temperature and proteinease du to the stabilising effect the circularisation confers (Perez et al., 2015). A very broad inhibition spectrum was observed when testing the inhibition potential of NKR 5-3B. As can be observed in results section 3.4, three indicators produced colonies that were resistant against the antimicrobial effect of the bacteriocin.

The strategy was to obtain and isolate several mutants that survived being subjected to the NKR 5-3B. This would allow a comparison between the wild type of *E. Thailandicus* with its mutant brethren, by a sequencing of their DNA. Any systematic deviation in the genetic material of the different strains of mutants, as compared to the wild type strain, would indicate areas that could be involved in the production of structures utilised by the bacteriocin to kill the target cell. Re-insertion of missing genes by plasmid into some of the mutated strands of *E. thailandicus* could potentially make them vulnerable to the NKR 5-3B again, thus providing a sturdy platform on which to theorise causality for the role of the receptor in this process.

More often than not, it is expected that obstacles emerge to upset an experimental model like the one outlined above. Relatively early in the project, the indicator cells' resistance proved unstable to maintain when picked, isolated and tested again with the NKR 5-3B bacteriocin. Surviving cells would appear, but these did not give rise to a stable strain of mutants. What emerged was a new outcome of a few resistant cells, quite similar to the previous inhibition zone it was isolated from. This indicated that a stable change in the DNA of these indicator cells was not the case. There is reasons to suspect that these are adaptive cells. This entails that their resistance to the bacteriocin was a change in the gene expression pattern, and not because of a DNA mutation that is responsible for an eventual receptor that NKR5-3B uses. There were thus no mutants generated. Section 3.5 of the results part illustrates this. Without a stable selection of *E. thailandicus* mutants, the rest of this part of the project would come to a halt, since these are vital to illustrate the role of the receptors for the bacteriocin. There are previous studies that have observed cells that were impervious to the NKR 5-3B (Hololovina, 2018). But these could have been

adaptive cells instead of mutants, since this was not explored further.

What is obtained, are observations about the ability of the bacteriocin NKR 5-3B to prevent the emergence of resistant bacteria. Across the different strains tested, none generated mutants with stable resistance to the bacteriocin, but instead developed mere resistance among a few cells. Literature on the characteristics of circular bacteriocins such as NKR 5-3B shows that the mechanisms of these might be different than linear bacteriocins, such as Nisin A. Several circular bacteriocins display activity that suggest they do not interact with specific receptors. Instead, they bind to the cell membrane itself. The effect is that the cell membrane is permeabilised, disrupting the cell potential and causing leakage of different ions (Van Belkum et al., 2011). This kills the susceptible cell.

There is however, heterogeneity among the circular bacteriocins. Studies suggest that the circular bacteriocin Garvicin ML uses the maltose ABC transporter to exert its antimicrobial effect. Over a certain concentration of Garvicin ML, inhibitory effects seems independent on whether the receiving cell has the maltose transporter or not. This suggests two different effects operating at different concentrations (Gabrielsen et al., 2012). The findings in this project suggest that the NKR 5-3B could belong to the first category that solely functions through binding to the cell wall, independently of any specific receptor. Another possibility could be that there is a receptor involved, but that the receptor is simultaneously essential for the indicator's life functions. Mutants lacking the ability to produce the receptor would also not be viable, thus preventing their appearance in the test. However, this needs to be tested further in order to be determined.

4.2 Part 2

In this part of the thesis, a generalised goal of isolating bacteriocin-producers and characterising their bacteriocins was pursued. The source was 53 samples of raw milk delivered by the KBM department of Dairy Technology and Food Quality. This was done with two sets of indicator groups, the first with checking bacteriocin-effects against two indicators simultaneously, *Enterococcus faecalis* (B3330) and *Staphylococcus aureus* (B3325) in combination. The second set of indicators screened for effect against three single indicators *Streptococcus dysgalactiae* (B3890), *Staphylococcus aureus* (B3023), and *Enterococcus faecalis* (B2333). The result was no inhibition zones where the two indicators were used in combination. The three separate indicators generated findings,

however. Some of the screened agar plates gave rise to an abundance of inhibition zones, as shown in table 3.3. The samples were #385, #269 and #451. Out of these, 12 samples were chosen for further characterisation.

To identify the species of the strains, a 16S rRNA gene sequencing was performed. This revealed that sample #385 contained a heterogenous species mix, with *E. faecalis* and *S. uberis* being present. Sample #269 and #451 contained *S. dysgalactiae* and *S. parauberis*, respectively. An ERIC-PCR analysis was done to investigate whether there was difference between the five *E. faecalis* strains internally and similarly for the five *S. uberis.* If these were different, it would make sense to characterise the different strains as well. As figure 3.9 illustrates, the strains were not genetically different from each other. A logical next step was to choose one strain from each species. The choice fell on samples #13, #43, #269 and #451.

To find bacteriocin genes, samples of two *S. uberis*, two *E. faecalis* and the two unique strains were chosen for genomic sequencing. A Miseq (Illumina) platform was used to sequence the strains. The genomic data were then annotated by RAST and converted to FASTA files. With the help of BAGEL4, which is a bacteriocin gene mining tool, any genes matching already catalogued bacteriocin genes were displayed.
Sample #43 containing the *E. faecalis* displayed one putative gene for enterolysin A, and BLAST searches revealed a 100% identity. This is shown in figure 3.10. Since there was a complete match with the enterolysin A gene, it was decided that further characterisations such as mass spectroscopy and inhibition spectrum would not be necessary.
Sample #13 failed to reveal any bacteriocin genes. Since there was genetic homogeneity (based on the ERIC-PCR results) between the *S. uberis* in sample #8 and #13, the *S. uberis* from sample #8 was uploaded on BAGEL4 instead. The *S. uberis* strain had a more complex display of bacteriocin genes based on the BAGEL4 analysis, shown in figure 3.11. Bovicin, penocin A, a bacteriocin-like peptide and a putative bacteriocin of class IIc, were all registered as matches in the genome of this *S. uberis* strain. The BLAST matches were not very high for most of them, with the “putative bacteriocin” having the highest identity of 80.6%.

Proteinase K tests showed that the isolate from *S. uberis* was heat stable and subject to degradation. This gives reason to believe the antimicrobial is proteinaceous in nature. The enterolysin A was not heat stable. Enterolysin A is a class III bacteriocin, and as such is

heat-labile (Nilsen et al., 2003). This further supports the findings from genomic sequencing that showed a gene for this bacteriocin.
During the purification process, it was prudent to use the nutrient medium best suited for optimal production of bacteriocins. Figures 3.4 and 3.5 shows the antimicrobial effect over time by nutrient medium for isolates from sample #13 and #43. For the enterolysin A the clearly best choice was GM17, and time was best between 23 and 28 hours. The isolate from sample #13 had also Todd-Hewitt (TH) chosen as an alternative, since it caters to *Streptococci* nutritional needs. Interestingly, the best choices were BHI and GM17, and both showed decrease in activity when 45 hours had passed. The peak activity for both BHI and GM17 was after 21.5 hours.

Both enterolysin A from sample #43 and the antimicrobial supernatant from the *S. uberis* #13 were purified by cationic exchange chromatography and reverse phase chromatography. Ammonium sulphate precipitation preceded these steps.
MALDI-TOF mass spectroscopy was conducted with the bacteriocin produced from sample #13 *S. uberis*, in order to compare the mass of any significant peaks with the theoretical mass from the sequence found in the BAGEL4 program. The theoretical mass of the peptide indicated by BAGEL4 was 4860.5 Dalton after the leader peptide was removed. Findings from the mass spectrometry did not match this. There was one recurring peak in the samples, and the size was 2213.4 Dalton. This is a quite small peptide. It did not match any of the putative bacteriocins from the BAGEL4 analysis. A search through BACTIBASE based on the peptide size did not result in any similarities either.

The inhibitory profile of the *S. uberis* in sample #13 further revealed the characteristics of the antimicrobial peptide produced. Against some central pathogens, the antimicrobial peptide had high activity against *L. monocytogenes*, *S. uberis* and *E. faecium*. As important, *E. coli* and *B. cereus* were not affected in the test. In the mutual inhibition test displayed in figure 3.11, the *S. uberis* also inhibited other *Streptococci*. Given that most bacteriocins are produced to compete with related bacteria competing within the same niche, this makes sense. These are also a natural part of raw milk, given that the original samples were collected from these. Therefore, applications in treatment of mastitis seem like a potential application of this antimicrobial peptide.
In any case, a clear answer to the identity of the peptide produced by the isolate from sample #13 remains to be revealed. A few observations can nevertheless be made. The

peptide has most likely a mass of 2213.4 Dalton, and is thus very small. It seems almost to occupy the upper thresholds of the thiopeptide size domain as they are described as having up to 17 amino acid residues (Bennalack et al., 2014). On the other hand, the inhibition profile shown in section 3.18 and 3.19 reveals a great potential for *Listeria* and *Enterococcus* inhibition. This is very similar to the inhibition pattern of pediocins, as they inhibit similarly as the findings in sample #13 (Papagianni & Anastasiadou, 2009). In effect, this peptide inhibits like pediocins, but is of a very small size. This illustrates the need for laboratory techniques that go beyond genetic sequencing. The genes behind the peptide produced by the *S. uberis* isolate have not been identified, and only further hands-on laboratory techniques will do that.

5.0 Future work

The Covid-19 pandemic of 2020 has also impacted the universities, where students have been restricted in access to laboratory procedures. Thus, more could have been done to reveal more about the antimicrobial peptides in this thesis.

It is obvious that this project has some possibilities for completing its lines of inquiry. The most glaring need is in relation to the peptide with mass of 2213.4 Dalton produced by *S. uberis*, from sample #13.

The Amino acid sequence would be extremely useful, as the masses found by MALDI-TOF have not clearly found the bacteriocins predicted by the genetic sequencing in the results section 3.12. This can be done by Edman degradation, in which amino acids are removed from the N-terminal one at a time, and are thus possible to identify (Konigsberg, 1967). By knowing the sequence of amino acids in the peptide it would be possible to look for the location of the gene in the sequenced genome as well.

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Appendix:

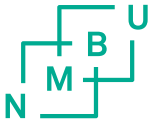
Table 1. Results of the antimicrobial spectrum for NKR 5-3B against 42 indicators. Points of 3 is two clear inhibition zones (maximum), while 0 is no inhibition.

Indicator	**Code**	**Bacteriocin zones of inhibition (average)**		
		NKR 5-3B	**Nisin A**	***Garvicin ML***
L. monocytogenes	B2653	1.5	2	2.5
L. monocytogenes	B2604	1	2	2
L. monocytogenes	B2650	1.5	2	2.5
L. monocytogenes	B2651	1	2	2.5
L. monocytogenes	B2652	1	2	2.5
L. innocua	B2710	1.5	2.5	2.5
L. innocua	B2785	1	2	2.5
L. Ivanovii	B2813	2	2	3
B. cereus	B2805	2	2	3
B. cereus	B2711	1	2	2
B. cereus	B2731	1.5	2	2.5
B. cereus	B2736	1.5	1.5	1.5
S. aureus	B3023	0	2	2
S. aureus	B3263	0	2	2
S. aureus	B3329	0	2	1.5
C. divergens (n=1)	*B2738*	1	2	2
E. faecalis	B2333	1	2	2
E. faecalis	B3088	1	2	2
E. faecalis	B3330	1	2	2
E. faecalis	B3331	1	2	2
E. faecalis	B3332	1	2	2
E. faecium	B2763	1	1.5	2
E. faecium	B2772	1	2	2
E. faecium	B2783	1	1	2
E. faecium	B2876	1	1.5	2
E. avium	B3465	2	3	3
L. plantarum	B2003	1	2	2.5
L. plantarum	B2352	1	2	3
L. plantarum	B3125	0.5	2	3

L. sakei	B2361	1.5	2.5	3
L. sakei	B2380	1	2	3
L. curvatus	B2353	1	3	3
L. curvatus	B2355	1	3	3
L. lactis	B1403	1.5	2.5	3
L. lactis	B1403	0	2	3
L. garvieae	B3390	2	3	3
S. thermophilus	B3555	2	2	2
S. uberis	B3912	1	3	3
S. dysgalactiae	B3890	1	2	2
L. gelidium	B2386	2	1	3
E. coli	B3591	0	0	0
E. coli	B3590	0	0	0

Table 2. Results of the antimicrobial spectrum for purified antimicrobial from sample #13 against 18 indicators. Points of 2 is two clear inhibition zones (maximum), while 0 is no inhibition.

Indicator	**Code**	**Bacteriocin zones of inhibition**		
		Sample 13	**Nisin**	***S. uberis* producer**
B. cereus	B2805	0	1.5	0
B. cereus	B2711	0	1.5	0
E. faecalis	B2333	0.5	2	0
E. faecalis	B3088	0.5	2	0
E. faecium	B2772	1.5	2	0
E. faecium	B2763	1.5	2	0
L. curvatus	B2355	1	2	1
L. garvieae (n=1)	B3390	1	2	0
L. monocytogenes	B2652	2	2	1
L. monocytogenes	B2651	2	2	1
S. aureus	B3263	0	1.5	0
S. aureus	B3325	0	1	0
S. dysgalactiae	B3890	0.5	2	1
S. uberis	B3912	2	2	1
S. uberis	B3920	2	2	0.5
S. uberis	B3980	2	2	1
S. uberis	B3918	2	2	0
E. coli (n=1)	B3590	0	0	0



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

Postboks 5003
NO-1432 Ås
Norway