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Screening for antimicrobial agents against the fish pathogens, *Streptococcus agalactiae* and *Yersinia ruckeri,* in fermented fruit and vegetables

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Christopher Heger

Abstract

Antibiotics have been used for a long period of time both for human and animal health, as well as economical benefits. Because of an emerging antibiotic resistance in pathogen microbes, there is an increasing need for new antimicrobials to control outbreaks of disease caused by pathogenic bacteria. One of these antimicrobials could be the group of antimicrobial peptides (AMP) called bacteriocins. These are peptides produced by bacteria in order to protect its niche from other competing bacteria.

The aim of this study was to search for bacteria able to produce bacteriocins inhibiting the two fish pathogens *Streptococcus agalactiae* and *Yersinia ruckeri* in samples made from fermented fruit and vegetables. This was done by screening, using a method based on separating the fish pathogen and the samples in different layers on an agar plate. Colonies from the samples that inhibited the growth of either of the fish pathogens were isolated and their bacteriocins were characterized by: spot-on-lawn inhibition assays, Sanger sequencing, REP-PCR, antimicrobial micro titer assays and mass spectrometry. 11 colonies were found producing bacteriocins against *S. agalactiae*. None bacteriocin producing bacteria were found against *Y. ruckeri*. All the 11 strains found when screening for *S. agalactiae* were shown to be *L. lactis* producing nisin Z. Purification of nisin Z produced by *L .lactis* in BHI and MRS resulted in more bacteriocins retrieved from a culture grown in BHI than in MRS. As a preliminary attempt for using *L. lactis* as a probiotic in aquaculture, we saw that our *L. lactis* was able to inhibit the fish pathogen when inoculated together in bottles of water.

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1 Introduction

Antibiotics has, since its introduction to clinical practice in the 1940s, been helping in controlling outbreaks of diseases caused by pathogenic bacteria (Aminov,2009). The use of antibiotics has in later years been applied in a lot of different areas including clinical practice, agriculture, animal farming and aquaculture, which has led to better health, for both humans and animals, as well as better economy (Aminov, 2009).

Most antibiotics are natural products disturbing important biochemical processes by targeting the bacterial cell wall, DNA or ribosomes (Brown, 2016). This results in inhibition of cell growth, cell division and in some situations cell death. Because antibiotics are natural products, defense mechanisms have appeared in the targeted bacteria. These mechanisms include: preventing entry of or exporting drugs, producing enzymes that destroy or modify the antimicrobial, or making changes to the antimicrobial target (Holmes et al., 2016). A consequence of the wide spread use of antibiotics has been the creation of an evolutionary selection pressure for bacteria containing antibiotic resistant mechanisms, leading to a higher prevalence of these bacteria (Davies and Davies, 2010, Michael et al. 2014).

In 2009, a database listed over 20 000 potential genes for antibiotic resistance of almost 400 different types predicted from bacterial genomes (Davies and Davies, 2010). These are frightening numbers which could be believed to increase in the following years. To add to this impending crisis, lack of new antibiotic discoveries in later time (between 1960s and the 1990s), a fear of going back to the prebiotic era has emerged (Xavier et al, 2016, Aminov, 2009, Brown et al., 2016).

1.1 Aquaculture and control of pathogenic bacteria

Antibiotics have also been used for controlling outbreaks of pathogenic bacteria causing damage and death to cultivated fish in aquaculture. Aquaculture produced 44% of the total fish production in 2014 (FAO, 2016). In numbers, this results in 74 million tons of fish with a value of 160 billion dollars, which was mainly used for human consumption (FAO,2016). Outbreak of diseases in fish farms is an ongoing issue which could cause several problems for the community. These include damage to the livelihood of farmers, loss of jobs, reduced incomes, and food insecurity (Assefa and Abunna, 2018). Almost 50% of the production loss found in fish farms has been shown to stem from disease outbreaks (Assefa and Abunna, 2018).

The use of antibiotics has been observed to cause negative effects in fish farms. Antibiotic resistant bacteria could cause severe damage in the fish industry, causing disease and death of the fish. There is also a fear for antibiotic resistant bacteria being transmitted to humans, for example by fish consumption. (Sequeiros et al., 2015). If these bacteria are human pathogens as well, this could result in severe damage to human health. An additional fear is that genes encoding antibiotic resistance could be transmitted to human pathogens by horizontal gene transfer (Sequeiros et al., 2015). In addition, antibiotics could alter both the natural fish gut microbiota and the general microbiota found in the area. This could lead to disturbance of the natural ecosystem which might lead to changes in fish nutrition, physiology and immunity (Banarjee and Ray, 2017 and Romero et al., 2012). These negative effects have led to several countries developing strict regulations when it comes to use of antibiotics in aquaculture, where only a few antibiotics are licensed for use (Romero et al., 2012, Rodgers and Furones, 2009). These regulations have resulted in a new focus on finding alternatives to the use of antibiotics in recent years.

Vaccines have been a popular method for preventing bacterial diseases in aquaculture. This comes from the lack of occurrence of drug resistance in vaccinated animals. In addition, it has been seen that nonvaccinated animals are protected from the diseases due to herd immunity (Assefa and Abunna, 2018, Romero et al., 2012). Because vaccines are only working as a preventive method, methods for controlling existing disease outbreaks are also needed.

An alternative for controlling outbreaks of bacterial disease could be the usage of probiotics. Probiotics are defined by WHO as “live microorganisms that when administered in adequate amounts, confer a health benefit on the host” (Romero et al., 2012). When we talk about probiotics to controlling outbreaks, we refer to the usage of bacteria, but also other microorganisms such as bacteriophages, microalgae and fungi have been explored for usage as probiotics (Hai, 2015). Probiotics are used in aquaculture, either as food additive or added directly into water. The addition of microbes comes with many complications, which makes the process of selecting bacteria for usage as probiotics a difficult process. The introduced bacteria must be non – pathogenic to host organism, humans and other aquatic organisms (Romero et al., 2012). In addition, it is important that the selected organism do not contain plasmidic antibiotic resistant genes, don’t causes damage to the environment, as well as being able to live in the environment where it is added (Romero et al., 2012).

1.2 Antimicrobial peptides and LAB Bacteriocins

A group of molecules which could be used as an alternative to antibiotics are antimicrobial peptides/proteins (AMPs). AMPs are small, evolutionary conserved molecules produced as a defense mechanism against a broad range of targets including fungi, viruses and bacteria (Zhang and Gailo, 2016). Over 5000 AMPs have been discovered, ranging between five to over a hundred amino acids long. The AMPs have been found produced by a diverse range of organisms ranging from prokaryotes to animals (Bahar and Ren, 2013). Insects and plants have been found to produce AMPs as antibiotics against pathogenic bacteria, while bacteria produce AMPs as a protection of its own niche from competing bacteria (Zhang and Gailo, 2016).

This study focuses on the group of AMPs produced by bacteria, the bacteriocins. Bacteriocins are small, ribosomal produced, multi-functional peptides, showing antimicrobial activity at certain concentrations (Chikindas et al., 2018). Both bacteriocins working against related species (narrow spectrum) and against other genera (broad spectrum) has been found (Sang and Blecha, 2008). Bacteriocins are produced both by gram positive and gram-negative bacteria. In this study we have been focusing on finding bacteriocins produced by gram positive bacteria, more specific by lactic acid bacteria (LAB).

LAB are gram positive, low-pH tolerant and non-sporing rods or cocci. The group is characterized by the production of lactic acid from glucose (Mokoena, 2017). They are facultative anaerobe, aerotolerant bacteria mainly obtaining energy by fermenting sugar by substrate level phosphorylation (Willey et al. 2014). Most LAB are Generally Regarded as Safe (GRAS) by the American Food and Drug Agency (FDA), and has been granted the Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) (Silva et al., 2018). These markings make LAB easier to introduce in food industry and makes LAB to one of the most used group of bacteria in industry.

1.3 Classification of LAB-bacteriocins

Different properties, such as producer organism, molecular weight, posttranslational modifications, and biological activity, could be used for classifying LAB bacteriocins. The first attempt was done in 1993 by Klaenhammer, ordering bacteriocins in four classes and several subclasses: Class I; lantibiotics, Class II; Small, heat-stable, non-lanthionine containing, membrane-active peptides, ClassIII; Large, heat-labile proteins and Class IV; Complex proteins. There have been several modifications to this classification. Some new

classifications continue using four classes; lantibiotics, non-modified non lantibiotics, large heat-labile bacteriocins and cyclic bacteriocins (Nes et Al. in 2007). Other classifies LAB bacteriocins after two classes: peptides with post-translational modification and peptides without modifications (Cotter, 2013).

In this thesis we followed a recent classification proposed by Alvarez-Sieiro et. Al in 2016. The classification is based on biosynthesis mechanism, post translational modifications and biological activity, including newly found bacteriocins that wouldn't fit in classification systems proposed earlier. This classification has three main classes divided in several subclasses. These Classes are: Class 1- ribosomal-synthesized and post-translationally-modified bacteriocins, Class 2- small, unmodified bacteriocins and class 3 – big, unmodified bacteriocins(Table 1-1).

Table 1-1: Overview of the classification of LAB bacteriocins based on a diagram from Alvarez-Sieiro et al., 2016

Class	**Description**	**subclass**	**Example**
Class I	Posttranslational modified, Heat stable peptides smaller than 10 kDa	1a lantibiotics	Nisin
		1b cyclized peptides	Enterocin AS-48
		1d linear azol(in)e-containing peptides	Streptolysin S
		1e glycocins	Glycocin F
Class II	Non modified, heat stable peptides smaller than 10 kDa	2a pediocin-like	Pediocin PA-1
		2b two-peptides	Lactococcin Q
		2c leaderless	Lacticin Q
		2d non-pediocin-like, single-peptide	Lactococcin A
Class III	Unmodified, thermolabile peptides larger than 10 kDa	bacteriolysins	Zoocin A
		non-lytic	Caseicin

Class I: small posttranslationally modified peptides

Class I consists of small(<10kDa), heat stable peptides undergoing enzymatic modifications during biosynthesis. These are subclasses of molecules existing in a group of molecules called ribosomally-synthesized and post-translationally-modified peptides (RiPPs) (Arnison et Al., 2013). The classification also contains two subclasses not included in this thesis: sactibiotics (subclass 1c) and lassopeptides (subclass 1f). These are chosen not to be included as no

bacteriocins from these classes has been characterized produced from LAB, only predicted or found in silico.

Class Ia contains the lantibiotics. These bacteriocins contain the thioether amino acids lanthionine and/or methyllanthionine. These are amino acids formed by post-translational modifications and result in intramolecular cyclic structures (Bierbaum and Sahl, 2009). The lantibiotics are divided into two subgroups: subgroup A and subgroup B. Subgroup A consists of elongated, cationic peptides containing up to 34 amino acids. Lantionine bridges are a common feature found for these bacteriocins. Nisin and Subtilin are examples of subgroup A lantibiotics. Subgroup B contains globular peptides up to 19 amino acids long. Duramycins are an example of subgroup B lanthibiotics (McAuliffe et al., 2001)

Class Ib consists of bacteriocins with cyclic structures. The cyclic structure is achieved by connecting the N and C terminal ends. Cyclic bacteriocins are often cationic and amphiphilic peptides consisting of five to six alpha helixes. Most of the observed cyclic bacteriocins has been produced by firmicutes (Montalbán-López, 2012). Two modes of action have been observed, both involving the making of pores (Alvarez-Sieiro et al., 2016).

Class 1d consists of linear azol(in)e-containing peptides (LAPs), peptides containing derivates from cysteine, serine, and threonine residues. Streptomycin S is the most known LAP.

Class Ie contains the glycosins, bacteriocins containing glycosylated residues. Enterocin F4-9 and glycocin F are characterized glycocins (Alvarez-Sieiro et al., 2016).

Class II: unmodified bacteriocins

Class II consist of small (<10kDa), heat stable peptides which do not undergo other posttranslational modifications other than cleaving off the leader peptide from the core peptide. Class II is built up of four subclasses.

Class IIa contains the pediocin-like bacteriocins. More than 50 bacteriocins of this type have been found, and the name comes from the first discovered bacteriocin in this group, Pediocin PA1. Pediocin-like bacteriocins are 6 to 49 amino acids long peptides. (Kjos et al., 2011) The peptides often consist of two parts, one N-terminus half containing a characteristic conserved motif (YGNGVXC) and a less conserved C-terminus most likely involved in target-cell specificity (Alvarez-Sieiro et al., 2016).

Class IIb consist of two-peptide bacteriocins. These are bacteriocins consisting of two peptides with different functions. Two kinds of two peptide bacteriocins have been found. The first is bacteriocins functioning only while both peptides are at presence, and the second one is peptides with enhanced function by the presence of both peptides (Alvarez-Sieiro et al., 2016).

Class IIc consist of leaderless bacteriocins, bacteriocins produced without a leader pepetide at the N-terminal. The leader peptide often functions as a navigator for bacteriocin secretion. Because of this it is likely that leaderless bacteriocins are dependent on a dedicated ABC transporter for secretion. Enterocin L50 produced by *E. faecium* is one of the most studied leaderless bacteriocins (liu, 2011, Alvarez-Sieiro et al., 2016).

Class IId: consist of non-pediocin-like, single-peptide bacteriocins. The bacteriocins found in this group are single linear peptide bacteriocins with different mechanisms for function and secretion. lactococcin 972, lactococcin A, and enterocin B Are examples of bacteriocins forund in this class (Alvarez-Sieiro et al., 2016).

Class III

Class III consists of bigger (>10kDa), thermo-labile peptides which do not undergo bigger modifications. Two subclasses are found in classIII: bacteriolysins and non-lytic class III bacteriocins (Alvarez-Sieiro et al., 2016).

1.4 Bacteriocin mode of action

Most bacteriocins produced by lactic acid bacteria work by creating pores of various sizes in the target's membrane (Kjos, 2011). Small weighted molecules like ions leaks out through the pores, resulting in reduction of the proton motive force damaging the cells. What separate the pore making bacteriocins from each other is which receptors they use as target molecules.

Type A lantibiotics have been showed to target Lipid II, a vital precursor in bacterial cell wall synthesis translocated across the phospholipid layer (Kjos, 2011). The best studied mechanism for pore formation using lipid II as target molecule is the mechanism found for Nisin. Pores made by nisin and lipid II are formed by Nisin binding to Lipid II via the lantibiotic ring structures found at the N-terminal (Bierbaum and Sahl, 2009).

Another well-established target for pore formation by LAB bacteriocins is the mannose phosphotransferase system (Man-PTS) receptors (Kjos, 2011). It is proposed that Class IIa, like pediocin PA1 kills its target by using these molecules as targets to form pores.

Even though the use of pores is the most observed mode of action of LAB-bacteriocins, other mechanisms has also been found. For example, has subgroup B lantibiotics been observed inhibiting cell wall synthesis (Bierbaum and Sahl, 2009).

1.5 Biosynthesis of bacteriocins

LAB bacteriocins are produced and matured in different manners but retains some common features. Common for all bacteriocins is that the bacteriocin-associated genes are arranged on the same locus. The genes can be organized in various ways, but all bacteriocin gene clusters consist of at least: structural genes, immunity gene(s) and genes needed for processing and transport(figure1-1). (Snyder and Worobo, 2013)

In most cases bacteriocins requiring post transcriptional modifications often have more complex genetic organization. The genetic structure of nisin is an example of this, consisting of 11 genes organized in three different operons (Snyder and Worobo, 2013). The structural gene (*NisA*) and two genes needed for posttranslational modification (*NisB* and *NisC*) are located in one operon, the second encoding a translocating protein (*NisT* and the immunity protein (*NisI*) and the last operon encodes three genes encoding an ABC transporter for protein secretion (*NisF*, *NisE* and *NisG*). (Snyder and Worobo, 2013 and AlKhatib et al., 2014) The gene organization found for the class IIa bacteriocin Pediocin PA1 is an example of less complex gene structure. Pediocin PA1 consists of one operon containing four genes: the structural gene (*papA*), a gene encoding the immunity protein (*papB*), a gene encoding a protein for cleaving the leader peptide (*papC*) and a gene encoding a transporter for secreting the bacteriocin (*papD*) (Kotelnikova and Gelfand, 2002, Snyder and Worobo, 2013).

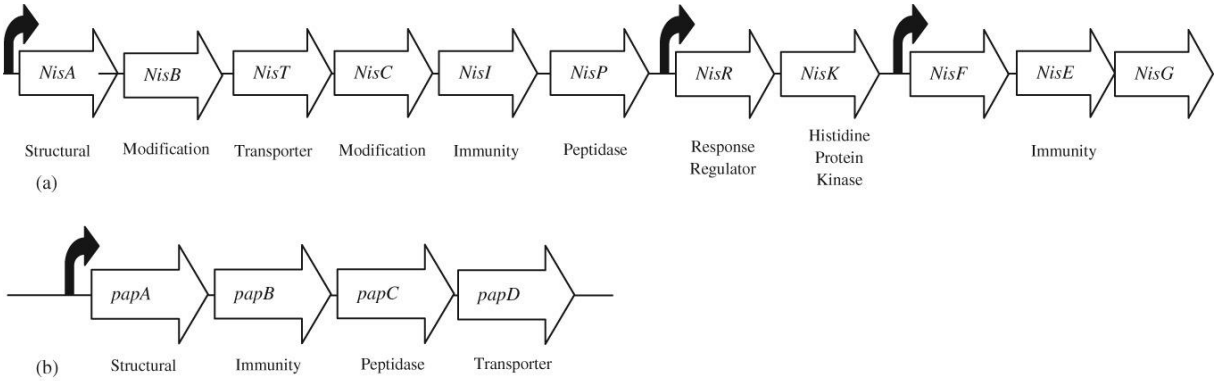

Figure 1-1 *Schematics of gene clusters found for (a) Nisin A (b) Pediosin PA1 (Snyder et. Al, 2013)*

To prevent the bacteriocins from damaging its producer it is important to have mechanisms for hindering bacteriocins from functioning while inside the producer and mechanisms to transport the bacteriocin out from the cell .

For obtaining self-immunity most bacteriocin gene clusters contain an immunity gene co-regulated with the structural gene (Kjos, 2011). The mechanisms of immunity vary for different bacteriocins and in many cases the actual mechanisms for immunity are uncharacterized. The best described immunity mechanism is the mechanism found for group A lantibiotics targeting lipid II molecules, where a combination of bacteriocins pumped out of the cell by a special ABC transporter and immunity proteins communicating with the bacteriocins outside the cell and in a way hindering the bacteriocins from targeting lipid II-targets on producer cell (Kjos, 2011).

It would not be beneficial for the bacteria to produce a high amount of bacteriocins at all time. Because of this, the bacteria have developed different ways for regulating the production, so that more bacteriocin is produced when the bacteriocin is needed. These mechanisms often depend on either signal molecules resulting in quorum sensing or a form for stress response (Kjos, 2011). In Gram positive bacteria quorum sensing is the regulation method most often observed, differentiated by using different types of molecules as signal molecules. (Snyder and Worobo, 2013). Lantibiotics have been observed using its own bacteriocin as regulator, for example nisin, which has been proved to function both as bacteriocin and as a signal molecule inducing the production. (Kleerebezem et al., 1997)

1.6 Application of bacteriocins

The use of bacteriocins in the food industry has been explored in the recent years. Most often bacteriocins have been used in purified form as a food preservative. Other methods of use include adding bacteriocin-producing strains directly in food as starter or protection cultures and adding bacteriocins during packaging of products (Ross et al., 2002, Snyder and Worobo, 2013, Perez et al.,2014). Another aspect with bacteriocins is that they have simpler biosynthetic mechanisms compared to antibiotics, making them easier to bioengineer, in order to increase activity or specify target organism. (Perez et al., 2014)

The first commercially approved bacteriocin was the lantibiotic Nisin. Nisin was marketed in England already in 1953 and assessed to be safe for food use by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives in 1969. Now it is approved for use in over 48 countries (Ross et al., 2002,

Snyder and Worobo, 2013). Nisin has been used to improve shelf life of a broad range of products worldwide, ranging from processed and cottage cheese to dairy desserts and liquid egg. It has also been used to inhibit spoilage bacteria during beer and wine fermentations, and the exploitation of nisin-producing strains has been shown to improve different kind of vegetable fermentations. (Ross et al., 2002).

Some bacteriocins have been shown to work synergistically when used together with other antimicrobial agents (Chikindas et al., 2018, Garshalloui et al., 2015, Mathur et al., 2017). It is proposed that this synergy is due to the antimicrobials is speeding up each other's inhibiting effect, resulting in reduced likelihood for obtaining resistance against the antimicrobials (Mathur et al., 2017). Nisin has for example been observed getting an enhanced effect when combined with other molecules such as lysozyme, essential oils and lactates (Garshalloui et al., 2015).

Several attempts have been done in trying to apply bacteriocin producing bacteria as probiotics in animal systems. It has been observed that gram positive bacteriocin producers could be found in theGastrointestinal (GI) tract of humans and animals (Gillor et al., 2008). For example, a study found a strain *Lactobacillus salivarius* producing a bacteriocin (Abp118). Further, these producers have been used to show survival against *Listeria monocytogenes* in studies done on mice, which could be related to the produced bacteriocin (Corr et al., 2007). Gram positive bacteria, including LAB, are also being used as probiotics in aquaculture (Hai 2015). Several of these have shown being producers of bacteriocins. Heo et al. (2012) isolated a nisin Z producing *L. lactis* from the intestines from olive flounder which was shown to inhibit the growth of the fish pathogen *Streptococcus iniae*. Since the bacteria already lived inside the fish as a part of its microflora, this is a bacterium that could be further tested for use as a probiotic bacterium.

Some disadvantages with the use of bacteriocins do also exist. Development of resistance against bacteriocins in sensitive bacteria has been observed (Kjos, 2011, Bastos et al.,2015). The frequencies and mechanisms of the resistance are varying, but it is thought that changes in the surface properties of the target cells could be the general way for obtaining resistance (Kjos, 2011).

1.7 Fish pathogens used in this thesis

In this study we will try to find bacteriocin producing bacteria against two fish pathogens: *Streptococcus agalactiae* and *Yersinia ruckeri*. These two bacteria are important pathogens

because they are pathogenic against Tilapia (Mian et al., 2009) and salmonids (Kumar et al., 2015).

Y. ruckeri is a gram-negative, rod-shaped, facultative anaerobe bacterium located in the Enterobacteriaceae family. *Y. ruckeri* is most known for being the causative agent of enteric red mouth disease in various species of salmonids (Kumar et al., 2015). The bacterium is found all over the world including the US, Canada, Europe, South America, the Middle East, China, India and Australia (Kumar et al., 2015).

S. agalactiae is a beta hemolytic, gram positive coccus. The bacterium causes Streptococcosis, a major bacterial disease in many fish species, mainly those cultivated in warm water. Observations have been made of the bacteria causing damage in both marine and fresh water, both In the wild and in fish farms. (Mian et al., 2009). Symptoms found in infected fish involves septicemia and bacterial colonization in different organs including the nares, brain, kidney and intestines. Clinical signs of streptococcosis, includes depression or excitability, anorexia, erratic swimming and whirling (Kannika et al., 2017)

S. agalactiae is especially important because it has outbreaks in the fish species Tilapia. In 2014 Tilapia was the third most produced fish in fish farms, only after carp and salmon, with over 3.5 million tons of fish produced (Munang'andu, 2016). Outbreaks of *S.agalactiae* could therefor lead to severe economic loss and less amount of food. S. agalactiae is also associated with diseases in humans, dogs, cows, horses and guinea pigs (Mian et al., 2009).

1.8 Aim of the thesis

This study had the aim of finding LAB producing bacteriocins against the two fish pathogens *S. agalactiae* and *Y. ruckeri*, which could be used in controlling disease outbreaks made by these bacteria in fish farms. Furthermore, we wanted to characterize the produced bacteriocins.

Figure 1-2 shows an outline of the work flow followed during this work, and which types of results that were obtained from each step. The work consisted of screening samples made of fermented fruit and vegetables, spot-on-lawn inhibition assays, squencing, fingerprinting, making of growth curves, production differences in different broths, purifying bacteriocin by chromatography, characterization of bacteriocin by mass spectrometry and an experiment for testing if the bacteriocin inhibitied growth of the pathogen when grown in water.

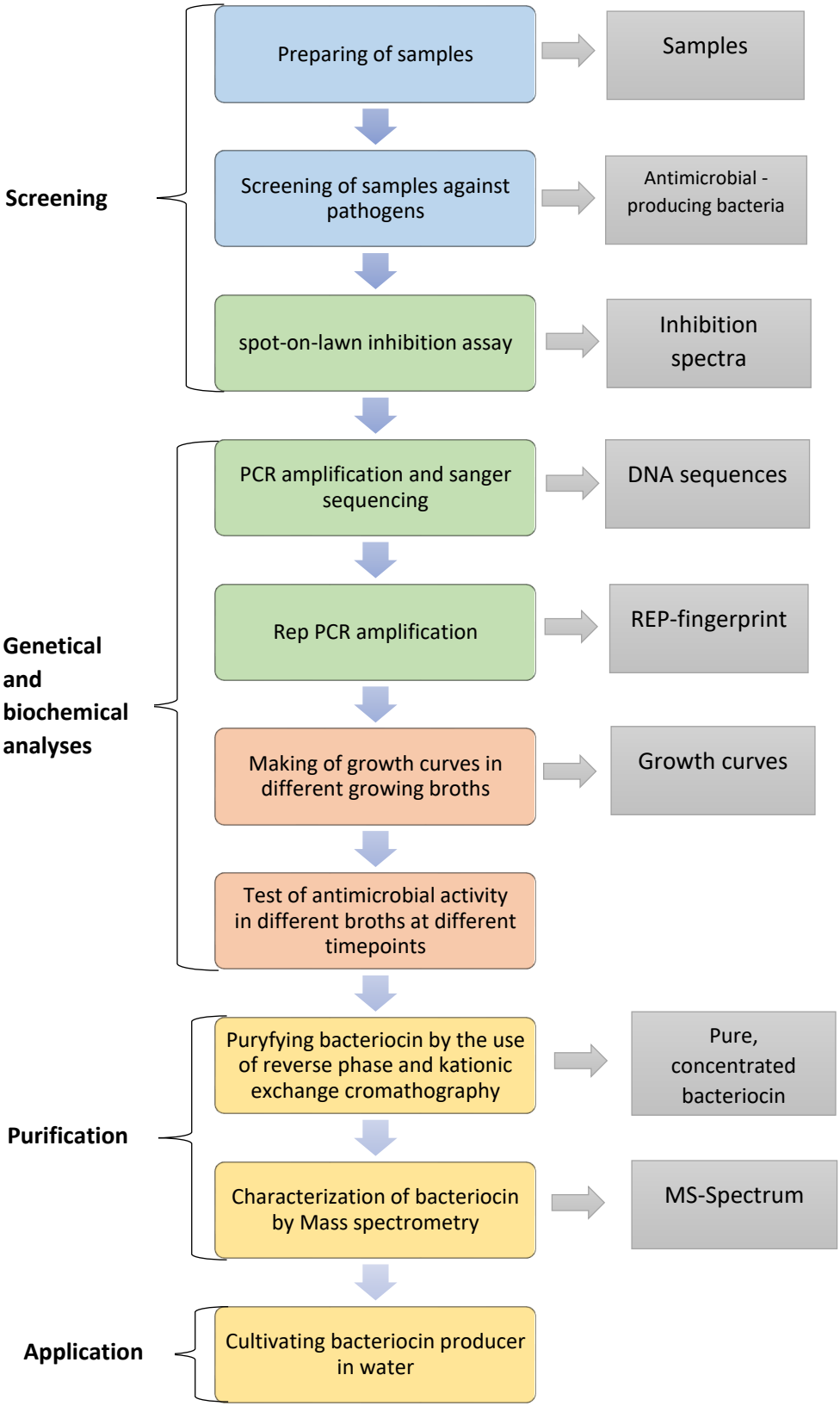

Figure 1-2 *Flowchart of the workflow in this study. The blue part was done using both S. agalactiae and Y. ruckeri as indicator bacteria. The green part is done only with strains showing antimicrobial activity against S. agalactiae. The red part is done only for strains showing different REP-fingerprint. The orange part is only done using a selected strain, strain 35. The grey parts indicate which results that was gathered from the step*

2 Materials and methods

2.1 Growth conditions

Special growth media are needed when working with bacteria. In this study, five different media have been used: de Man, Rogosa and Sharpe (MRS)(OXOID), Brain Heart Infusion (BHI)(OXOID), GM17 (OXOID), Lysogeny broth (LB)(OXOID) and Edwards medium Broth (OXOID). The media were prepared following the recipes given by the manufactures. The media has been used as liquid growth medium, soft agar and solid agar. Solid agar and soft agar were made containing respectively 1,5% and 0,8% agar powder

Bacteria used in this study were Lactic acid bacteria (LAB) and the fish pathogens *S. agalactiae* and *Y. ruckeri*. MRS growth medium was used for optimizing nutrition conditions for LAB. LAB was grown in an incubator at 30°C. Both the fish pathogens were grown in BHI broth. *S. agalactiae* was grown in incubator at 37°C and *Y. ruckeri* at room temperature(~20°C).

Procedures with a high risk of being contaminated have been performed with sterile, autoclaved equipment's and disposable gloves in sterile work benches with fume hood.

2.2 Collection of samples

Two collections of samples made from fermented fruit and vegetables were used in this study. These samples were provided by supervisor with permission to use. The samples were made by taking 20- 50 g of the fruit/vegetable in a container(bag/cup) together with tap water. The containers were stored in an outdoor storage room for fermenting. After fermenting for the desired time, 1 ml of sample liquid was squeezed out, made glycerol stock of and stored at -80°C until further use.

The first collection of samples (table 2-1) was made from 24 different fruits and vegetables bought from a Norwegian convenient store. Samples were prepared after both one and two months of fermenting.

The second collection of samples (table 2-2) was made from 50 different fruits and vegetables bought at a Turkish shop in Hauketo, Oslo. Two containers were prepared for each fruit/vegetable, one with and one without added salt. This was done to make different environments for bacteria growth. The samples were then fermented for three weeks.

Table 2-1 List of fruits and vegetables included in the first sample collection

Sample ID	Source	Sample ID	Source
1	Blue berry	13	Celery
2	Dried tomato	14	Fennel
3	Plum	15	Sweet potato
4	Beetroot	16	Leek
5	Parsnip	17	Brocoi
6	Celeriac	18	Onion
7	Dark beet	19	Apple
8	Light beet	20	Rutabaga
9	Strawberry	21	Carrot
10	Raspberry	22	Cauliflower
11	Salad	23	Potato
12	Isbergsalat	24	Pear

Table 2-2 List of fruits and vegetables included in the second sample collection.

Sample ID	Source	Sample ID	Source
1	Green grapes	27	Kelek/ Turkish Cucumber
2	Blue grapes	28	Romanesco broccoli
3	Orange watermelon	29	Sugar pees
4	Small cucumber	30	Aristo
5	Sweet cherries	31	Chinese cabbage
6	Avocado	32	Quince (kvede
7	Pineapple	33	Fichianindia
8	Purple aubergine	34	Rambutan
9	Apricot	35	Eddo/Taro
10	Raspberries	36	Fig
11	Blueberries	37	Green, small mango
12	Tomatoes	38	Sweet potato
13	Mango	39	Lychee
14	Blue plum	40	Sharon
15	Ladyfinger/Okra	41	Aubergine
16	Dates	42	Large Chili
17	Passion fruit	43	Kiwi
18	Strawberries	44	Red Onion
19	Plums	45	Blackberry
20	White aubergine	46	Rotten apples from garden
21	Physalis	47	small, green chili
22	Pear	48	Dragon fruit (Tanh long)
23	Karela/Balsam pear	49	Jackfruit
24	Taro/Eddo roots	50	Longgong
25	Chayote/Chow chow	51	Apples
26	Banana		

2.3 Screening for antimicrobial producing bacteria

A screening method selective for LAB bacteria was chosen (figure 2-1) and executed over a time period of three days (figure 2-2). It was carried out in two rounds, one for *S. agalactiae as* indicator bacterium and one for *Y. ruckeri*. The protocol was the same for both indicator bacteria, except for different incubation temperatures for the two pathogens, 37 °C for *S.agalactiae* and room temperature for *Y. ruckeri.*

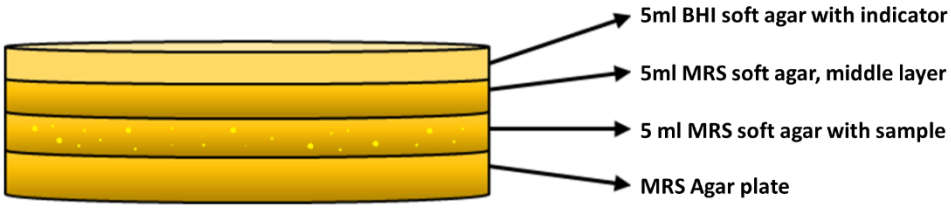

Figure 2-1 *An overview of the different layers in the screening method.*

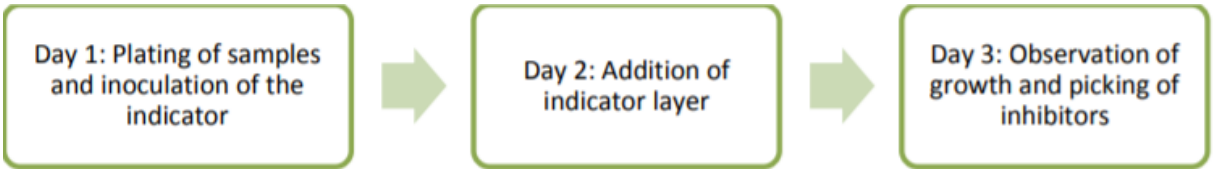

Figure 2-2 *Flowchart of the screening method (Haldorsen, 2017).*

The samples were diluted using 0.9% NaCl and sterile test tubes containing 5 ml of melted MRS soft agar held at 48°C (figure 2-3). The dilutions were poured over MRS agar plates and left to solidify. A second layer of 5 ml MRS soft agar was poured over the plates and was left for solidifying. This layer worked as a middle layer hindering mixing and smearing between the colonies from the samples and the indicator bacteria added the next day. The plates were placed in anaerobic growing chambers. AnaeroGen™ bags (Thermo Scientific) were used for creating an anaerobic environment, which is favorable for LAB. The plates were incubated over night at 30 °C.

The second day, a top layer containing the indicator bacterium was added to the plate. Overnight (ON) culture of the indicator was diluted 25 times in melted BHI soft agar (500 µl ON culture in 100 ml BHI soft agar). A layer of 5 ml melted BHI soft agar, containing indicator bacterium, was poured on the MRS plates and left for some time for the soft agar to

solidify and dry. The plates were incubated at 37°C for the indicator *S. agalactiae* and at room temperature for *Y. ruckeri*.

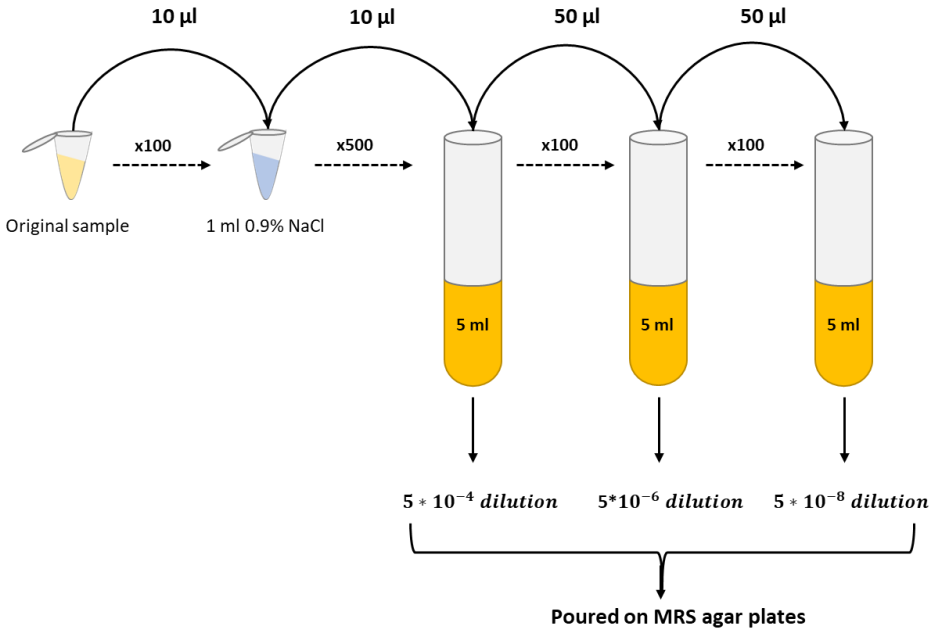

Figure 2-3 *Dilution of samples resulting in three dilutions:* $5 * 10^{-4}, 5 * 10^{-6}$ *and* $5 * 10^{-8}$.

On the third day, the plates were checked for growth and inhibition zones. Colonies inhibiting the growth of the indicator bacteria were picked using a sterile toothpick, streaked on MRS agar plates and incubated ON at 30°C. Pure cultures of the colonies were made by picking single colonies with sterile toothpicks, inoculating in 5 ml MRS broth and incubating ON at 30°C. Glycerol stocks were made by adding 15% glycerol to the ON culture and were frozen at -80°C for further use.

Samples showing minimal or little growth were tried enriched. The enrichment was done by inoculating 100 µl sample in Sterile test tubes containing 5ml liquid MRS medium and incubated ON at 30°C. using this method, the little numbers of bacteria found in the samples could grow further in the medium. The following day, the enriched samples were treated as ordinary samples, and were screened for by following the same protocol as just described.

2.4 Spot-on-lawn inhibition assay

The pure cultures obtained from the screening were further tested for purity and antimicrobial activity by a spot-on-lawn inhibition assay. Supernatants from the pure cultures were obtained by centrifuging 1,5 ml of ON- cultures at 13 rpm for 10 minutes. The supernatant was filtered through 0,45 mm filters. All the supernatants were in addition treated with heat treatment and with proteinase K. Heat treatment was performed by heating 100 µl supernatant in a heat

block at <90 °C for 10 minutes. The Proteinase K treatment was executed by mixing 20 µl supernatant with 20 µl proteinase K in an Eppendorf tube placed in a water bath at 37 °C for an hour.

BHI agar plates were prepared for the spot-on-lawn inhibition assay by adding a layer of 5 ml BHI soft agar added 25 µl ON culture of the specified indicator bacterium. Drops of 10 µl supernatant obtained from ON cultures of the pure cultures were placed on marked spots on the prepared plates. The plates were incubated ON at 37°C for *S. agalactiae* and at room temperature for *Y. ruckeri.* The plates were observed for antimicrobial activity the next day. One parallel was made for each of the differently treated supernatants.

Spot-on-lawn inhibition assays were also carried out by using a library of 46 different bacteria strains in addition to a nisin producing strain *L. lactis* as indicators in order to check the inhibition spectra of the produced bacteriocin.

2.5 DNA methods

DNA-extraction

In order to perform genomic analysis of the pure cultures obtained from the screening, DNA had to be isolated. This was done by the use of a DNA extracting kit (GenElute Bacterial Genomic DNA kit).

For harvesting cells, a volume of 1,5 ml ON culture of the strains were centrifuged at 13 000 x g. The cells (the pellet) were resuspended in 200 µl prepared lysosome solution and incubated in a water bath at 37 °C for 30 minutes (the supernatant was discarded). Afterwards, 20 µl proteinase K solution and 200 µL lysis solution C were added to the cells, mixed thoroughly and incubated in a water bath at 55°C for 10 minutes.

Pre-assembled GenElute Miniprep Binding columns were seated in collection tubes and marked for further identification. The columns were prepared for the DNA extraction by adding 500 µl column preparation solution to each column. The columns were centrifuged at 12 000 x g for 1 minute. The eluate was discarded and the column ready to use.

The lysate was prepared for binding to the columns by adding 200 µL of 96% ethanol. The whole lysate content was transferred to the column and centrifuged at 6500 x g for 1 minute. The collection tube was discarded, and the column was seated in a new collection tube. 500 µL wash buffer was added to the column and it was centrifuged for 1 minute at 6500 x g. The collection tube was discarded, and the column sat in a new collection tube. A second wash

was done by adding 500 µL washing buffer to the column and then centrifugated for 3 minutes at 13000 x g. The columns were centrifugated one extra minute at 13000 x g in order to get the column completely dry and free from ethanol. The collection tube was discarded, and the column placed in a new collection tube. 200µL of elution solution was placed directly in the center of the column, which was centrifuged at 13 000 x g for 1 minute. The elution should then contain the isolated DNA.

Nano drop

NanoDrop ND-1000 (NanoDrop Technologies) was used to quantify and measure the purity of the DNA. Nanodrop measures the absorbance at $\lambda = 260$ nm, the wavelength best absorbed by nucleic acids, and transform the absorption into concentration of DNA.

In addition to DNA concentration, nanodrop measures two ratios indicating the purity of the DNA extraction. A 260/280 ~ 1.8 indicates pure DNA, while lower values than 1.8 indicates contamination of proteins, phenol or other molecules which absorbs good at $\lambda = 280$ nm. A low 260/230- value indicates presence of contaminants absorbing well at wavelengths at 230 nm such as EDTA, carbohydrates and phenol. A low 260/230 ratio could also indicate problems with the sample or problem with the extraction method used. High 260/230-values indicates problems with the used blank, either by use of dirty column when measuring or the use of an inappropriate blank solution.

2.6 PCR (Polymerase Chain Reaction)

Polymerase chain reaction (PCR) was used to obtain a higher concentration of DNA fragments needed for further analysis. PCR amplifies specified regions of the DNA by the use of specific primers. Three variants of PCRs were used during the study: a regular PCR amplification of the 16S-rRNA gene using OneTaq DNA polymerase (New England Biolabs), a regular amplification of the nisin gene using Phusion® DNA polymerase (New England Biolabs), and a repetitive element palindromic-PCR (rep-PCR) using OneTaq DNA polymerase. An overview of all the used primers can be found in table 2-3

Table 2-3 *Primers used in this study*

Primer	Sequence
16S-12R	5’-AGGGTTGCGCTCGTT-3’
16S-11F	5’-TAACACATGCAAGTCGAACG-3’
nisZ-R	5’-GGATAGTATCCATGTCTGAACTAAC-3’
nisZ-F	5’-CTACAAAATAAATTATAAGGAGGCACTC-3’
REP-1R	5’-IIIICGICGICATCIGGC-3’
REP-2I	5’-ICGICTTATCIGGCCTAC-3’

PCR amplification of the 16S-rRNA, using Specific primers (16S -12R and 16S 11F) (table 2-3), was performed to make taxonomical assignments down to genus level. 16S-rRNA is RNA from small ribosomal sub units, integrated in the structure of the ribosome. Ribosomes are crucial for the survival of the bacteria leading to a slow rate of changes in the sequences in this region. Slow rate of change has led to highly conserved molecules where the differences can be used to differentiate between organisms. The molecule contains both sequences variating among related organisms, used to compare close related organisms, and sequences similar for related organisms, allowing comparison of distantly related organisms.

A regular PCR amplification using specific primers for the nisin gene (nisZ-R and nisZ-F) (table 2-3) was made to check if the isolated strains contained the gene coding one of the forms of the bacteriocin nisin. Because the different nisin's varies in just some amino acids Phusion DNA polymerase was used instead of one taq polymerase for getting a more accurate sequence.

REP-PCR is a method where primers (REP-1R and REP-2I) (table 2-3) match to repetitive extragenic palindromic (REP) regions of varying sizes found in the genome. When running the PCR products on an Agarose gel electrophoresis, a fingerprint pattern is formed consisting of the amplified REP-regions. These fingerprints can be used to differ strains from each other.

16S-PCR

A PCR reaction mixture was made by mixing nuclease free water, OneTaq buffer (New England Biolabs), nucleotides (new England Biolabs), primers and Taq DNA polymerase according to table 2-4. A volume of 49.5µl of the master mix was mixed with 0,5µl of genomic DNA in a PCR tube. The PCR tubes were placed in a PCR Thermocycler (Bio-Labs) and ran according to table 2-5.

Table 1-4 *Composition of Master mix used in the 16S PCR amplification*

Components	50 µl RXN	Final concentration
5X One Taq standard reaction buffer	10 µl	1X
10mM dNTPs	1 µl	200µM
10 µl 11F Forward Primer	1 µl	0,2µM
10 µl 12R reverse Primer	1 µl	0,2M
Template DNA	0,5 µl	>1,000 ng
One taq DNA polymerase	0,25 µl	50µl PCR
Nuclease-free water	Up to 50 µl	

Table 2-5: *The program used for the amplification of the 16S-rRNA-gene.*

Temperature	Duration	cycles	Action
94 °C	30 seconds	1	Initial denaturation
94 °C	15 seconds		Denaturation
60°C	30 seconds	34	Primer annealing
68°C	30 seconds		Primer extension
68°C	5 minutes	1	Final extension
4°C		hold	storing

PCR of the nisin gene

A PCR reaction mixture was made by mixing nuclease free water, Phusion buffer (New England Biolabs), nucleotides, primers and Phusion DNA polymerase according to table 2-6. A volume of 49.5µl of the master mix was mixed with 0,5µl of genomic DNA in a PCR tube. The PCR tubes were placed in a PCR Thermocycler (Bio-Labs) and ran according to table 2-7.

Table 2-6 *Composition of Master mix used in the nisin gene PCR amplification*

Components	50 µl RXN	Final concentration
5X Phusion HF buffer	10 µl	1X
10mM dNTPs	1 µl	200µM
10 µl Nis z Forward Primer	2.5 µl	0,5µM
10 µl Nis z reverse Primer	2.5 µl	0,5M
Template DNA	0,5 µl	>1,000 ng
Phusion DNA polymerase	0,5 µl	3%
Nuclease-free water	Up to 50 µl	

Table 2-7 The program used for the amplification of the Nisin-gene

Temperature	Duration	cycles	Action
98 °C	30 seconds	1	Initial denaturation
98 °C	10 seconds		Denaturation
59°C	30 seconds	34	Primer annealing
72°C	1 minute		Primer extension
72°C	5 minutes	1	Final extension
4°C		hold	storing

REP-PCR

A PCR reaction mixture was made by mixing nuclease free water, OneTaq buffer, nucleotides, primers and Taq DNA polymerase according to table 2-8. A volume of 49.5µl of the master mix was mixed with 0,5µl of genomic DNA in a PCR tube. The PCR tubes were placed in a PCR Thermocycler (Bio-Labs) and ran according to table 2-9.

Table 2-8 The composition of the master mix used in REP-PCR amplification

Components	50 µl RXN	Final concentration
5X One Taq standard reaction buffer	10 µl	1X
10mM dNTPs	1 µl	200µM
10 µl Rep 1forward Primer	1 µl	0,2µM
10 µl REP 2I reverse Primer	1 µl	0,2M
Template DNA	0,5 µl	>1,000 ng
One taq DNa polymerase	0,25 µl	50µl PCR
Nuclease-free water	Up to 50 µl	

Table 2-9 Program used for the REP-PCR amplification

Temperature	Duration	cycles	Action
95 °C	7 minutes	1	Initial denaturation
94 °C	1 minute		Denaturation
41°C	1 minute	35	Primer annealing
65°C	3 minutes		Primer extension
65°C	16 minutes	1	Final extension
4°C		hold	storing

2.7 Agarose Gel electrophoresis

Agarose gel electrophoresis is performed to visualize the PCR products. During gel electrophoresis, the PCR products are applied to an agarose gel at a constant electric field. DNA is negatively charged and will travel towards the positive charge in the electrical field. Because of pores in the agarose gel, smaller DNA-molecules will travel faster trough the gel than bigger molecules, allowing DNA fragments to be differentiated by size.

The agarose gel was made with 1% agarose for PCR products after regular PCR amplifications and with 1,8% for running the REP-PCR products. Agarose was added to 50 ml 1X TAE buffer and the mixture was boiled in the microwave oven until the agarose was totally dissolved. The gel was added 2µl Peqgreen DNA dye (PEQLAB), mixed, and poured into a mold. A comb was placed in the mold to make wells, so that the PCR products could be applied to the gel. The gel was left for 30-40 minutes for solidification and placed in an electrophorese chamber. The first well was applied with 7 µL ladder and the rest with 10 µL sample mixed with 2 µL loading buffer. The gel was run for 40 minutes at 90V for the regular amplification products and 180 minutes at 80V for the REP-PCR products.

The gels were visualized with UV-light by BIO RAD molecular imager. Gel images are made by DNA dye binding to the DNA fragments resulting in DNA fragments visualized as bands when exposed to UV-light. The sizes of the fragments are measured by comparison against a ladder with fragments of known sizes.

2.8 Purification and sequencing of PCR products

The PCR products had to be purified before they could be sent for sequencing. Purification of the PCR products was done using "Nucleospin Gel and PCR Clean-up" – clean up kit. The cleanup was done after following protocol:

The PCR products were added two times the original volume of NTI-buffer and the mixture was mixed well. The mixtures were transferred to columns seated in collection tubes and centrifuged at 11 000 x g for 30 second. The eluate was discarded and washed by adding 700 µL NT3 washing buffer. The columns were centrifuged at 11000 x g for 30 seconds. The eluate was discarded, and the washing step performed once more. After the second wash the eluate was discarded, and the empty columns were centrifuged at 11000 x g for 1 minute for drying. The DNA was then eluted by adding 30 µL of elution buffer to the column followed by centrifugation at 1 minute at 11000 x g.

The DNA-concentrations of the purified PCR products were measured using Nano drop, and the DNA was prepared for sequencing. Two eppendorf tubes for each sample were prepared, one containing 5µl of 5µM forward primer, and one tube containing 5µL of 5µM reverse primer. 5 µl DNA was then added to each tube and sent for sequencing by the company GATC/ eurofins.

2.9 Time depended antimicrobial activity

Experiments were done to check if growth and antimicrobial activity varied when grown in different broths. In addition, growth and antimicrobial activity were tested for strain specificity. Growth was examined by the making of growth curves, while the activity was measured by Spot-on-lawn inhibition assays and antimicrobial micro titer assays.

Growth curves

Growth curves were made by inoculating ON culture of the strains in four different broths; MRS, BHI, GM17 and LB. Cell density in the ON cultures were estimated by measuring optical density (OD) at λ =600 nm. The OD was used to calculate necessary amount for obtaining an initial OD of 0,05 in a final volume of 200 µl. The calculated amount ON culture was added to wells in a microtiter plate containing the different broths such that a final volume of 200 µl was obtained. The microtiter plate was incubated at 30°C in a spectrophotometer (SPECTROstar Nano) measuring OD_{600} each 10 minutes for 18 hours. The OD readings were used for plotting growing curves for the strains grown in each broth.

Antimicrobial production over time

OD_{600} was measured of ON cultures of the selected strains. The OD was used for calculating how much ON culture was needed for obtaining an initial OD_{600} on 0.05 in 50 ml broth. The calculated amount ON culture was added to a falcon tube containing a volume of broth resulting in a final volume of 50 ml. The falcon tubes were incubated at 30°C. After 3, 6, 10 and 24 hours after inoculation, the OD_{600} was measured, and 1 ml aliquots were taken from each culture.

Supernatants of the aliquots was gathered by centrifuging the aliquots. The supernatants were filtered by 0,45mm filters, heated for 10 minutes at >90°C and stored at -20°C for later use. All the supernatants were tested for antimicrobial activity against *S. agalactiae* both qualitative and quantitative.

Antimicrobial activity was tested qualitatively using spot-on-lawn inhibition assays. For the spot-on-lawn inhibition assay BHI agar plates were prepared by adding a top layer of 25 times diluted ON culture of *S. agalactiae* (25 µl ON *S. agalactiae* culture added to 5 ml BHI soft agar). Droplets of 5µl supernatant was dropped on marked spots on the prepared BHI agar plates. The plates were incubated at 37 °C ON and checked for inhibition zones the following day.

Antimicrobial activity was tested quantitatively by use of antimicrobial micro titer assays. All wells in a micro titer plate were added 100 µl of BHI broth. 100 µl of supernatant was added to the first well in each line. Twofold dilutions were made between each well in a row starting with well 1 ending with well 10. 100 µl of 25 times diluted ON culture of the indicator bacteria was added to each well from the first to the eleventh. The 11. Column worked as negative controls and the 12. column worked as positive control controls. The plates were incubated at 37°C for 3-4 hours. The cell density was measured by spectrophotometer at λ = 600nm.

The antimicrobial activity detected in the antimicrobial micro titer assays was expressed as bacteriocin units (BU) per milliliter. A bacterial unit is defined as the least diluted dilution with the ability to inhibit growth of the sensitive indicator bacterium. A well was defined inhibited of bacteria growth if the measured OD was less than half the measured OD of the negative control.

2.10 Purification and characterization of bacteriocin

Protein purification was done to purify the produced nisin and to see if it was possible to retrieve a higher amount of bacteriocin from a culture grown in MRS broth than in BHI broth. The purification was done by cationic exchange chromatography and reverse phase chromatography following the steps given in the flowchart in figure 2-4.

Before the chromatography steps were performed, the proteins in the supernatant obtained from the colonies were concentrated using ammonium sulphate precipitation.

Cationic exchange is a form for ion exchange were cationic ions are separated from non-cationic molecules. This is done using a column containing ion exchange resins which are molecules with acidic functional groups bound to cationic ions. These cationic ions are then exchanged with cationic ions in the liquid transferred trough the column, which are later released in an elution liquid.

Reverse phase chromatography is a separation method where a polar mobile phase is going through a non-polar stationary phase. The molecules in the mobile phase are binding to the stationary phase and is later eluted at different pace, where the more polar molecules are faster eluted than the less polar.

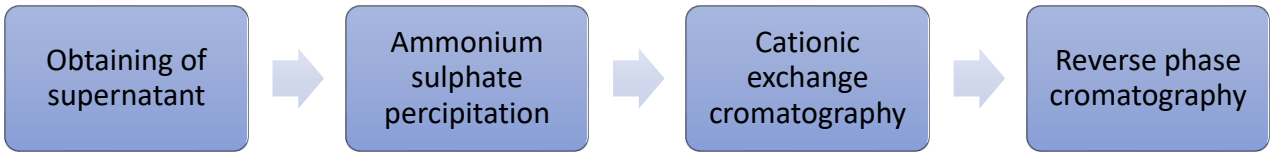

Figure 2-4 *Flowchart of the steps made during nisin purification*

Obtaining of supernatant and Ammonium sulphate precipitation

It was inoculated 13 ml of ON culture of *L.lactis* (strain 35) in 487 ml of growth broth for obtaining an initial OD_{600} on 0,05. The cultures were grown for 10 hours, the optimal growth time found by the antimicrobial assays, at 30°C. After 10 hours incubation the cells were centrifuged at 9 000 x g at 4°C in 30 minutes. The supernatant was poured over in a new bottle and the cell pellet was discarded. An aliquot of 1,5 ml of the supernatant was taken out, filtered and frozen at -20°C for later use. 150 g of ammonium sulfate was added to each 500 ml of supernatant, calculated by the ammonium sulphate calculator http://www.encorbio.com/protocols/AM-SO4.htm, and shaked thoroughly. The supernatants were stored at 4°C for 24 hours.

The ammonium sulfate solutions were spun down for 45 minutes at 9 000x g at 4 °C. The supernatant was discarded, and the protein pellet resuspended in 100-150 ml distilled water. An aliquot was taken, filtered and frozen at -20°C for further use. The pH for the solutions was measured and adjusted to pH≈4 with 1M HCl. The pH adjustment was done in order to better the conditions for applying the solution to the column during cationic exchange. Additional precipitation after the pH adjustment was removed by centrifuging once more at 9 000 x g for 15 minutes. The supernatants were now prepared for cationic exchange chromatography.

Cationic exchange chromatography

The cation exchange column HIPrep 16/10 SP-XL column (GE Healthcare Biosciences) was prepared by equilibration with water at pH 4 made by adding 1 M HCL to distilled water. The protein solution was then applied on the column with a flow rate of 10 ml/ minute. The

column was washed with approximately 100 ml of 20 mM phosphate buffer (pH=6.9) until the column was clean. 100 ml of 0.5 M sodium chloride was applied to the column at a speed of 5 ml/ minute to elute the bacteriocin. To ensure the release of all proteins bound to the column, 100 ml 2.0 M NaCl was sent through the column with a speed of 10 ml/minute. The flow through, wash liquid, elution and 2.0M NaCl elution were stored at 4 °C for later use. The original supernatant, ammonium sulphate precipitation, flow through, wash liquid, elution liquid and 2 molar NaCl liquid were tested for antimicrobial activity using antimicrobial micro titer assay with *S. agalactiae* as indicator. This was done in order to ensure that the steps done until this point had worked according to plan and was executed as described in the section 2.9.

Reverse phase chromatography

The bacteriocin was further purified and concentrated by reverse phase chromatography on a resource RPC column by ÄKTA purifier system (Amersham Pharmacia Biotech). The column was equilibrated by running water at pH 4 through the column until the pH and conductivity were stable. The eluate from the cationic exchange was then applied to the column. The column was washed with mobile phase A (water containing 0,1%TFA) until no proteins went through to the flow through. A linear gradient of mobile phase B (isopropanol containing 0,1% TFA) was then applied with a flow rate at 1.0 ml/min. Two attempts were done for each medium with different gradients used in the different attempts in order to increase the quality of the purification.

Absorbance at λ = 280 nm and λ =214 nm were measured during the elution. This was to indicate the presence of proteins eluted at given time during the chromatography. The wave lengths were chosen, because 214 nm is the wave length absorbed best by peptide bonds and 280 nm being the best absorbed wave length for aromatic bonds.

The elution liquid was collected in test tubes containing fractions of 1 ml each. For examine which fractions was containing the bacteriocin, each fraction was qualitatively tested for antimicrobial activity in a micro titer plate. This was done by adding 10 µl of each fraction to 190 µl 50 times diluted ON culture of *S. agalactiae* in wells on the microtiter plate. The plate was incubated at 37°C for three hours. OD_{600} was measured, and the wells with a significantly low OD were treated as active fractions.

The active fractions were tested in an antimicrobial micro titer assay with *S. agalactiae* as indicator. 20 µl of the active fractions were mixed with 180 µl BHI in the first well in each

row on a microtiter plate. 100 µl BHI broth was added to the rest of the wells on the plate. A dilution series was made between well 1 and 10 with two-fold dilutions between each well. 100 µl 25 times diluted *S.agalactiae* was added in each well from column one to eleven. Column 11 worked as negative control and Column 12 as a positive control. The plates were incubated at 37°C for 3 hours, and OD_{600} was measured by a spectrophotometer and BU/ml was calculated.

Characterization of nisin by the use of mass spectrometry

The most active fraction from the second purification attempt from both broths were sent for mass spectrometry. This was done for checking the purity of the fractions, to confirm that the isolated bacteriocin indeed was Nisin Z and checking if post translational modifications were present.

2.11 Cultivating the nisin producer and S. agalactiae in water

S. agalactiae and *L. lactis* (strain 35) were cultivated in water to test if *L. lactis* was able to inhibit the growth of *S. agalactiae* in water. Four bottles were prepared by mixing components according to table 2-10 and incubated at 30 °C. Yeast peptone was added for giving the bacteria an amount of nutrition for growth. Aliquots were taken and spread on agar plates after 0, 10 and 24 hours after inoculation. In order to get a countable number of bacteria on the plates, the aliquots were diluted in a micro titer plate(fig.2-5) before plating. Plates made of Edwards medium, a selective medium for *S. agalactiae*, and MRS, common medium for growth of LAB, were used in this experiment. The plates were incubated for 24 hours at 30°C and the colonies were counted in order to calculate colony forming units (CFU).

Table 2-10 *Preparation of the bottles for inoculation of bacteria in water. ON cultures of the specified bacteria were used*

Bottle ID	Components
A	45 ml tap water, 5ml 1x yeast-peptone, 50 µl *L. lactis*
B	45 ml tap water, 5ml 1x yeast-peptone, 50 µl *S. agalactiae*
C	45 ml tap water, 5ml 1x yeast-peptone, 50 µl *L.lactis*, 50 µl *S. agalactiae*
D	45 ml tap water, 5ml 1x yeast-peptone,

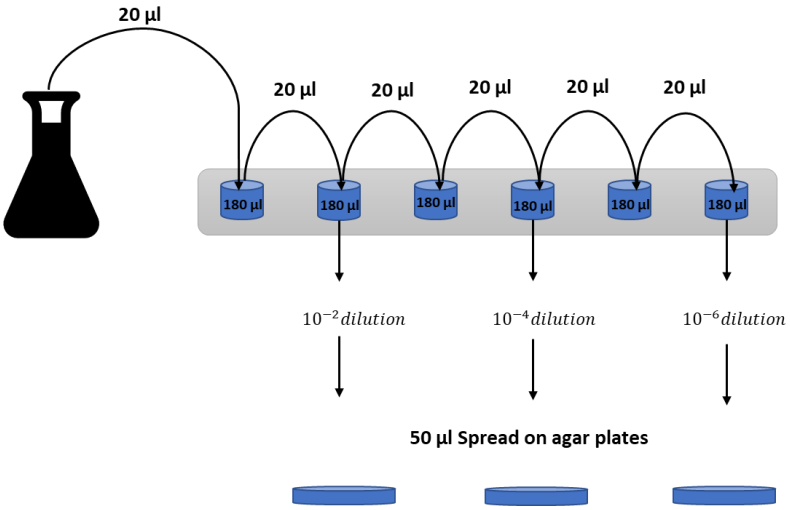

Figure 2-5 *An overview of how the dilutions made of each of the bottles during the water experiment were prepared. The dilutions were made by diluting with distilled water added 10% yeast peptone.*

3 Results

3.1 Screening for bacteria with antimicrobial activity

A collection consisting of 24 samples made from different fermented fruits and vegetables was screened for antimicrobial activity against the fish pathogens *S. agalactiae* and *Y. ruckeri*. A low cell number was found in most of the samples and the initial dilution step in NaCl was skipped when screening these samples. Some colonies were causing small, weak inhibition zones, but this was likely caused by lactic acid produced by the bacterium. Unfortunately, no bacteria showing antimicrobial activity were found for either of the two fish pathogens.

An enrichment of the samples was performed but had little effect. No bacteria with antimicrobial activity was found after this attempt.

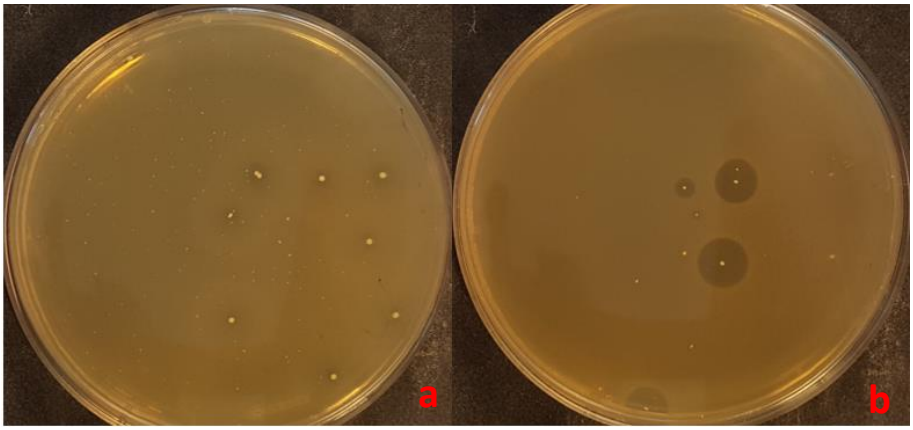

Figure 3-1: *colonies showing antimicrobial activity. a) the dilution* 10^{-4} *of sample 44 against S. agalactiae. Zones likely caused by lactic acid. b) the dilution* 10^{-6} *of sample 48+ against S. agalactiae inhibtion zones caused by bacteriocin producing bacteria.*

An additional screening of a second library of samples made from 50 different fermented exotic fruits and vegetable was done. The samples showed a varying cell number (figure 3-1), where the number of colonies seen in the dilutions varied from >300 to 1 colony. 14 colonies were isolated from eight different samples during the screening against *S. agalactiae* (table 3-1). No colonies with antimicrobial activity were found against *Y. ruckeri*.

Table 3-1 Colonies showing antimicrobial activity against the fish pathogen S. agalactiae

sample	Source	Colonies picked	Strain names
6	Avocado	3	6a, 6b, 6c
8	Purple aubergine	1	8
30	Aristo	1	30
35	Eddo/taro	1	35
42	Large chili	2	42a, 42b
44	Red onion	1	44
48	Dragonfruit	2	48a, 48b
48+	Dragonfruit	3	48+a, 48+b, 48+c

3.2 Spot-on-lawn inhibition assay

Spot-on-lawn inhibition assays were used to test antimicrobial activity of the pure cultures against the *S. agalactiae*. Additionally, a library of 46 different strains plus a *L. lactis* strain producing Nisin were used as indicators for testing the inhibition spectra of the strains.

Most of the strains showed antimicrobial activity in the supernatant both without (figure 3-2) and with heat treatment (figure 3-3). None of the strains had antimicrobial activity in the supernatant treated with proteinase K. However, the strains 6c, 44a and 44b showed no signs of antimicrobial activity in neither the supernatant with or without heat treatment. These strains were thus discarded and removed from further work. Some of the inhibition zones were observed containing single colonies growing inside the inhibition zone.

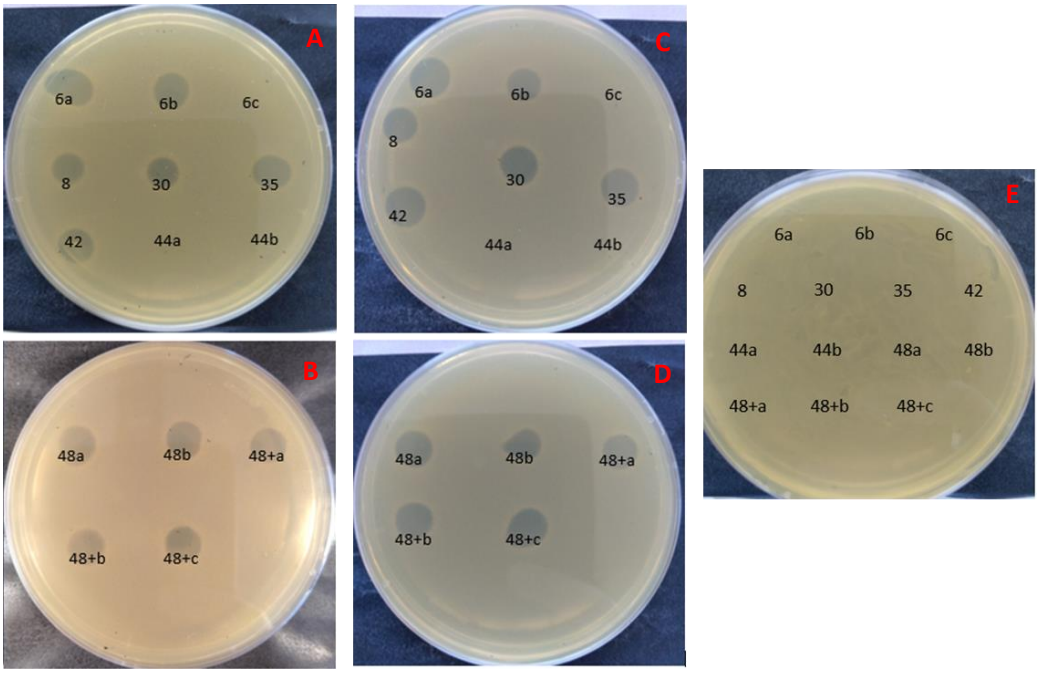

Figure 3-2 Spot-on-lawn inhibition assay of the isolated strains against S. agalactiae using: supernatant without treatment(A and B), supernatant with heat treatment (C and D), supernatant after proteinase K treatment (E)

To examine the inhibition spectra of the isolated strains, spot-on-lawn inhibition assays were also done with 46 different strains as indicator bacteria (table 3-2). All the strains seemed to have a high and similar range of inhibition. The strains had a similar inhibition spectrum found from a producer of the bacteriocin nisin Z, with some differences. In addition, none of the strains inhibited the growth of the nisin producing *L. lactis.* This indicated the possibility of the strains producing a variant of the bacteriocin nisin.

Table 3-2 *Antimicrobial activity shown by the supernatant from selected strains against 46 different strains. The inhibition spectrum observed for a producer of nisin (nis) is also included.*

Species	Strain nr	6a	6b	8	30	35	42	48a	48b	48+a	48+b	48+c	nis
Bacillus cereus	**LMGT2805**	+	+	+	+	+	+	+	+	+	+	+	+
Bacillus cereus ATCC 9139 B	**LMGT2711**	+	+	+	+	+	+	+	+	+	+	+	+
Bacillus cereus 1230, Granum 11-91	**LMGT2731**	+	+	+	+	+	+	+	+	+	+	+	+
Bacillus cereus ATCC 2 (matforsk)	**LMGT2736**	+	+	+	+	+	+	+	+	+	+	+	+
Carnobacterium divergens NCDO 2306	**LMGT2738**	+	+	+	+	+	+	+	+	+	+	+	+
Carnobacterium pisciola	**LMGT2332**	+	+	+	+	+	+	+	+	+	+	+	+
Enterococcus avium	**LMGT3465**	+	+	+	+	+	+	+	+	+	+	+	+
Enterococcus facecalis	**LMGT2333**	+	+	+	+	+	+	+	+	+	+	+	+
Enterococcus facecalis	**LMGT3088**	+	+	+	+	+	+	+	+	+	+	+	+
Entereococcus facecalis 158B	**LMGT3330**	+	+	+	+	+	+	+	+	+	+	+	-
Enterococcus facecalis 111A	**LMGT3331**	-	+	+	+	+	+	+	+	+	+	-	-
Enterococcus facecalis 29C	**LMGT3332**	-	+	+	+	+	+	-	+	+	+	-	-
Enterococcus faecium	**LMGT2763**	-	+	+	+	+	+	+	+	+	+	-	+
Enterococcus faecium	**LMGT2772**	-	+	+	+	+	+	+	+	+	+	-	+
Enterococcus faecium	**LMGT2783**	-	+	+	+	+	+	-	+	+	+	-	+
Enterococcus faecium	**LMGT2876**	-	+	+	+	+	+	-	+	+	+	-	+
Lactobacillus curvatus	**LMGT2353**	-	+	+	+	+	+	+	+	+	+	-	+
Lactobacillus curvatus	**LMGT2355**	-	+	+	+	+	+	+	+	+	+	-	+
Lactobacillus plantarum	**LMGT2003**	-	+	+	+	+	+	+	+	+	+	-	+
Lactobacillus plantarum	**LMGT2352**	-	+	+	+	+	+	+	+	+	+	-	+
Lactobacillus plantarum	**LMGT3125**	+	+	+	+	+	+	+	+	+	+	+	+
Lactobacilus sakei	**LMGT2361**	+	+	+	+	+	+	+	+	+	+	+	+
Lactobacilus sakei	**LMGT2380**	+	+	+	+	+	+	+	+	+	+	+	+
Lactobacillus salivarius	**LMGT2787**	-	-	-	-	-	-	-	-	-	-	-	-
Lactococcus garviae	**LMGT3390**	+	+	+	+	+	+	+	+	+	+	+	+
Lactococcus lactis	**Il1403**	+	+	+	+	+	+	+	+	+	+	+	+
Lactococcus lactis	**LMGT2081**	+	+	+	+	+	+	+	+	+	+	+	-
Leuconostoc gellidium	**LMGT2386**	+	+	+	+	+	+	+	+	+	+	+	-
Listeria innocua	**LMGT2710**	+	+	+	+	+	+	+	+	+	+	+	+
Listeria innocua	**LMGT2785**	+	+	+	+	+	+	+	+	+	+	+	+
Listeria ivanovii	**LMGT2813**	+	+	+	+	+	+	+	+	+	+	+	+
Listeria monocytogenes	**LMGT2604**	+	+	+	+	+	+	+	+	+	+	+	+
Listeria monocytogenes	**LMGT2650**	-	-	-	-	-	-	-	-	-	-	-	+
Listeria monocytogenes	**LMGT2651**	-	-	-	-	-	-	-	-	-	-	-	+
Listeria monocytogenes	**LMGT2652**	+	+	-	+	-	+	+	-	-	-	-	+
Listeria monocytogenes	**LMGT2653**	+	+	+	+	+	+	+	+	+	+	+	+
Pediococcus acidilactici	**LMGT2002**	+	+	+	+	+	+	+	+	+	+	+	+
Pediococcus pentosaceus	**LMGT2001**	+	+	+	+	+	+	+	+	+	+	+	+
Pediococcus pentosaceus	**LMGT2366**	-	-	-	-	-	-	-	-	-	-	-	+
Staphylococcus aureus	**LMGT3022**	-	-	-	-	-	-	-	-	-	-	-	+
Staphylococcus aureus	**LMGT3023**	-	-	-	-	-	-	-	-	-	-	-	+
Staphylococcus aureus	**LMGT3224**	-	-	-	-	-	-	-	-	-	-	-	+
Staphylococcus aureus	**LMGT3262**	-	-	-	-	-	-	-	-	-	-	-	+
Staphylococcus aureus	**LMGT3263**	-	-	-	-	-	-	-	-	-	-	-	+

Staphylococcus aureus	LMGT3264	-	-	-	-	-	-	-	-	-	-	-	+
Staphylococcus aureus	LMGT3265	-	-	-	-	-	-	-	-	-	-	-	+

+ indicates inhibition of the indicator – indicates no inhibition of the indicator.

3.3 All isolates are Lactococcus lactis

Amplifying of the 16S rRNA gene was done for classyfying the picked strains down to specie classification. The 16S-amplification with the primers 12R and 11F was expected to produce DNA fragments with a size of 1064 base pairs. The gel picture (figure 3-3) after gelectroforesis of the 16S- PCR products was showing a DNA fragment with aproximately the expected size. The sequences of all the strains matched the genome of *L. Lactis* with a 99% sequence similarity.

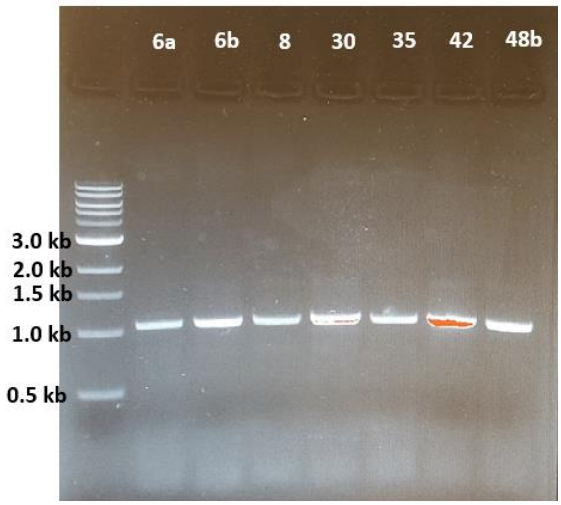

Figure 3-3 *The bands in some of the strains after amplification of the 16S-rRNA gene. A 1 kb ladder is used in the first well.*

3.4 The isolates are nisin producers

Previous results indicated that the isolated strains could be producers of the lantibiotic nisin. The nisin gene was amplified with specified primers for the nisin gene. The fragments formed by the selected primers were expected to have sizes of around 200 bp. The gel picture (figure 3-4) after gel electrophoresis showed that all the strains had a DNA fragment of the expected size. The sequences of the purified PCR-products confirmed that all the isolated strains had the gene encoding the bacteriocin Nisin Z (appendix A-1).

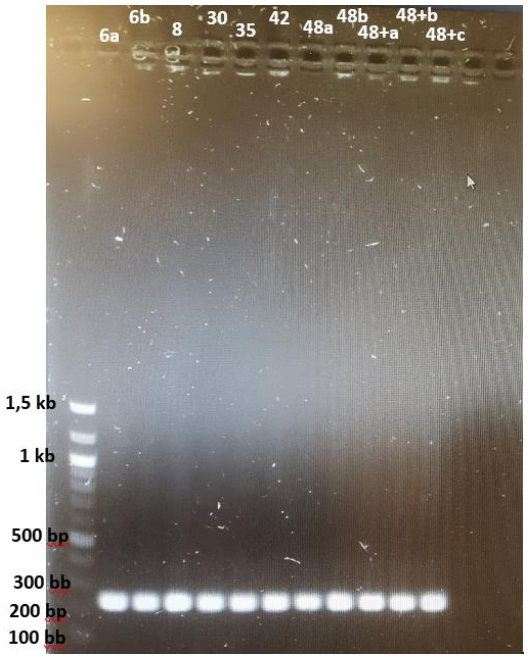

Figure 3-4: *Phusion PCR-bands of the amplification of the nis-gene from the isolated strains. A 100bp ladder was applied to the first well.*

3.5 REP PCR:

All the selected strains were fingerprinted by REP-PCR to look for genetic differences between the strains. It could be seen from the REP-PCR gel-picture (figure 3-5) that some of the strains shared the same REP-PCR fingerprint, but that most of them differed slightly from each other. In total, seven different fingerprints were found, and we decided to continue

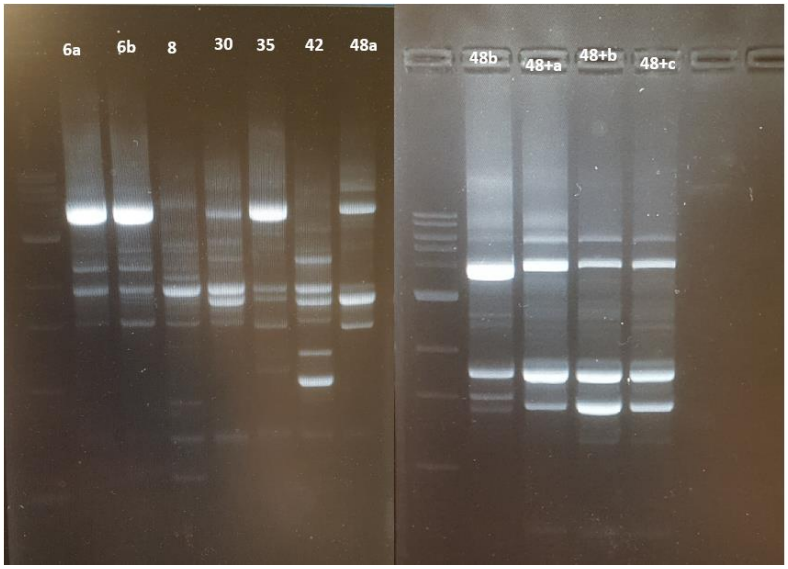

Figure 3-5: *REP-PCR results for the selected strains. A 1kb ladder is used in the first well on each of the gels.*

working with one strain representing each fingerprint. The selected strains were: 6a, 8, 30, 35, 42, 48b and 48+b.

3.6 Growth curves

We wanted to find out if the strains were growing differently in different broths. Growth curves (figure 3-6) were made of the different strains in MRS, BHI, LB medium and GM17. The growth curves were in general quite similar between the strains. BHI was containing the most growth, while the growth found in GM17 was similar but in general grew a bit slower than in BHI. MRS had a slower growth rate but reached the same OD as in in BHI and GM17 after around 10 hours of growth. LB had the poorest growth rate with a very low growth.

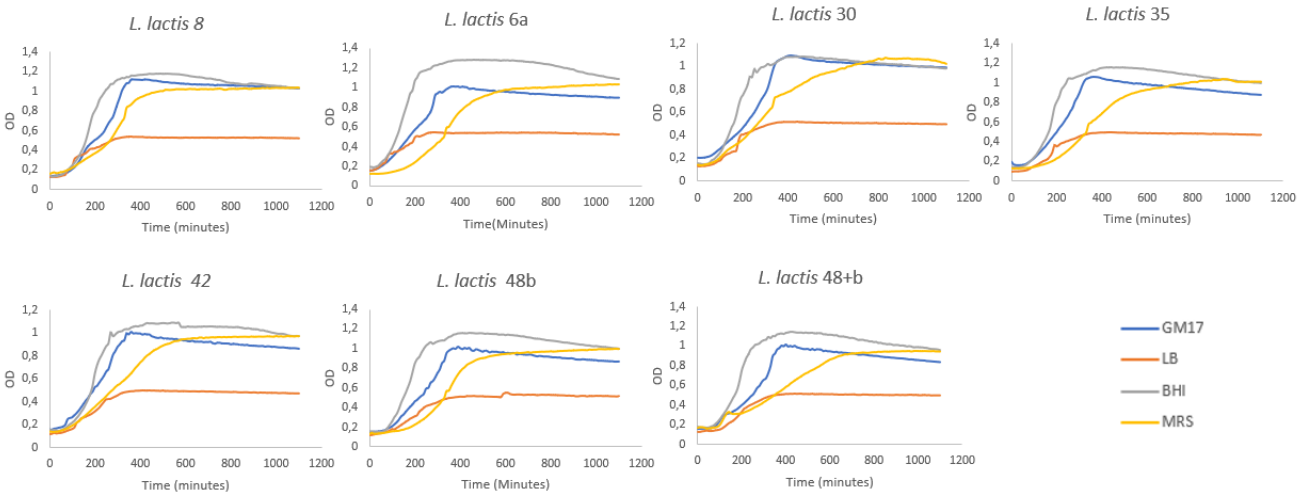

Figure 3-6 *Growth curves for the seven selected strains in different growing broths over 18 hours. Made by plotting OD_{600} against time*

3.7 Antimicrobial production over time:

Supernatants were gathered from cultures of the selected strains inoculated in the different broths at 3, 6, 10 and 24 hours. Antimicrobial activity was detected by both Spot-on-lawn inhibition assays and expressed in BU/ml by antimicrobial microtiter assays. This was done to get an overview of the antimicrobial activity at different time points after inoculation. Both the Spot-on-lawn inhibition assays (figure 3-7) and the antimicrobial micro titer assays (table 3-3) showed that the supernatants from the strains grown in MRS in general had more antimicrobial activity than the supernatants obtained from the strains grown in BHI, GM17 and LB medium. The supernatants obtained from the cultures grown in GM17 and BHI had the same rate of activity. It was observed that the strains had the highest antimicrobial activity after ten hours of growth.

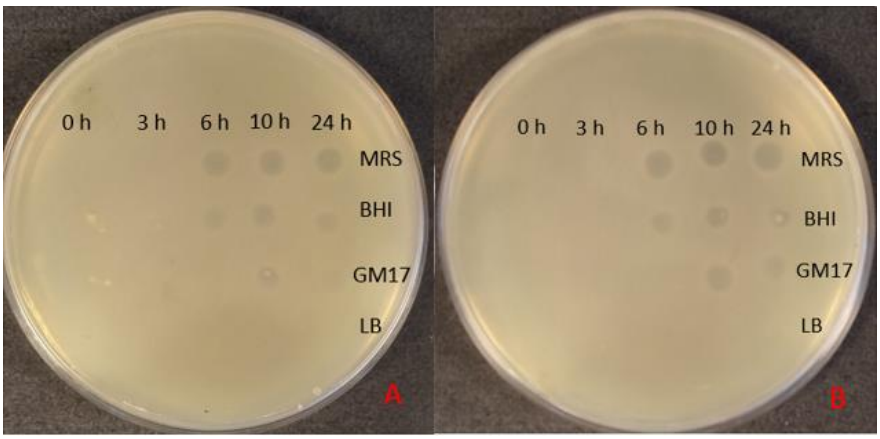

Figure 3-7 *Spot assay for sample 30 (A) and sample 35 (B) in different broths at different time points.*

Table 3-3 *Antimicrobial activity in supernatants obtained from cultures grown in different growth broths after 6, 10 and 24 hours of growth.*

	MRS			BHI			GM17		
Strain	**6h**	**10h**	**24h**	**6h**	**10h**	**24h**	**6h**	**10h**	**24h**
6a	40	40	40	20	20	20	20	20	20
8	20	40	40	0	20	20	0	20	0
30	40	80	40	20	40	20	20	20	20
35	40	80	80	20	40	40	20	20	20
42	20	40	40	20	20	40	20	20	20
48b	40	80	40	20	40	40	20	40	20
48+b	20	80	40	20	40	20	20	20	20

The results are given in BUs/ml

3.8 Purification of bacteriocin

From the results in 3.6 and 3.7, it was shown that the strains grew best in BHI, but seemed to have a higher antimicrobial activity in MRS. Bacteriocins were purified from cultures grown in both media to investigate if more bacteriocins could be retrieved from either of the media.

Differences in precipitation were observed in the different media. More proteins were visible precipitated in the supernatant obtained from culture grown in BHI than in supernatant from culture grown in MRS. This indicated that BHI contained more proteins in general than MRS.

Proteins eluted during the reverse phase chromatography were detected by measuring absorbance at $\lambda = 280$ nm and $\lambda = 214$. This is visualized in spectra made by ÄKTA (figure 3-8). The spectra confirmed that a lot more proteins are to be found in the BHI-solution than in

the MRS-solution. Even though the MRS contained a lower amount of proteins it could be seen that the purification for the MRS liquid was much better than the purification of the MRS liquid. Antimicrobial activity in the active fractions was expressed in BU/ml determined by antimicrobial micro titer assay. Table 3-4 and 3-5 summarizes the antimicrobial activity detected in each step of the purification process. By our calculations, it was retrieved almost the double amount of proteins from a culture grown in BHI than was retrieved for a culture of the same strain grown in MRS.

Table 3-3 *Antimicrobial activity in each step of the purification process of bacteriocin produced in BHI*

	V	Bu/ml	total BU	yield/ml, %	yield total,%
SN	500	40	20000	100	100
AS	140	160	22400	400	112
CE	100	160	16000	400	80
RP	8	2762,5	22100	6906,25	110,5

supernatant (SN), ammonium sulphate precipitation (AS), cationic exchange (CE) and reverse phase chromatography (RP).

Table 3-4 *Antimicrobial activity in each step of the purification process of bacteriocin produced in MRS*

	V	Bu/ml	total BU	yield/ml, %	yield total, %
SN	500	80	40000	100	100
AS	140	160	22400	200	56
CE	100	160	16000	200	40
RP	8	1500	12000	1875	30

supernatant (SN), ammonium sulphate precipitation (AS), cationic exchange (CE) and reverse phase chromatography (RP).

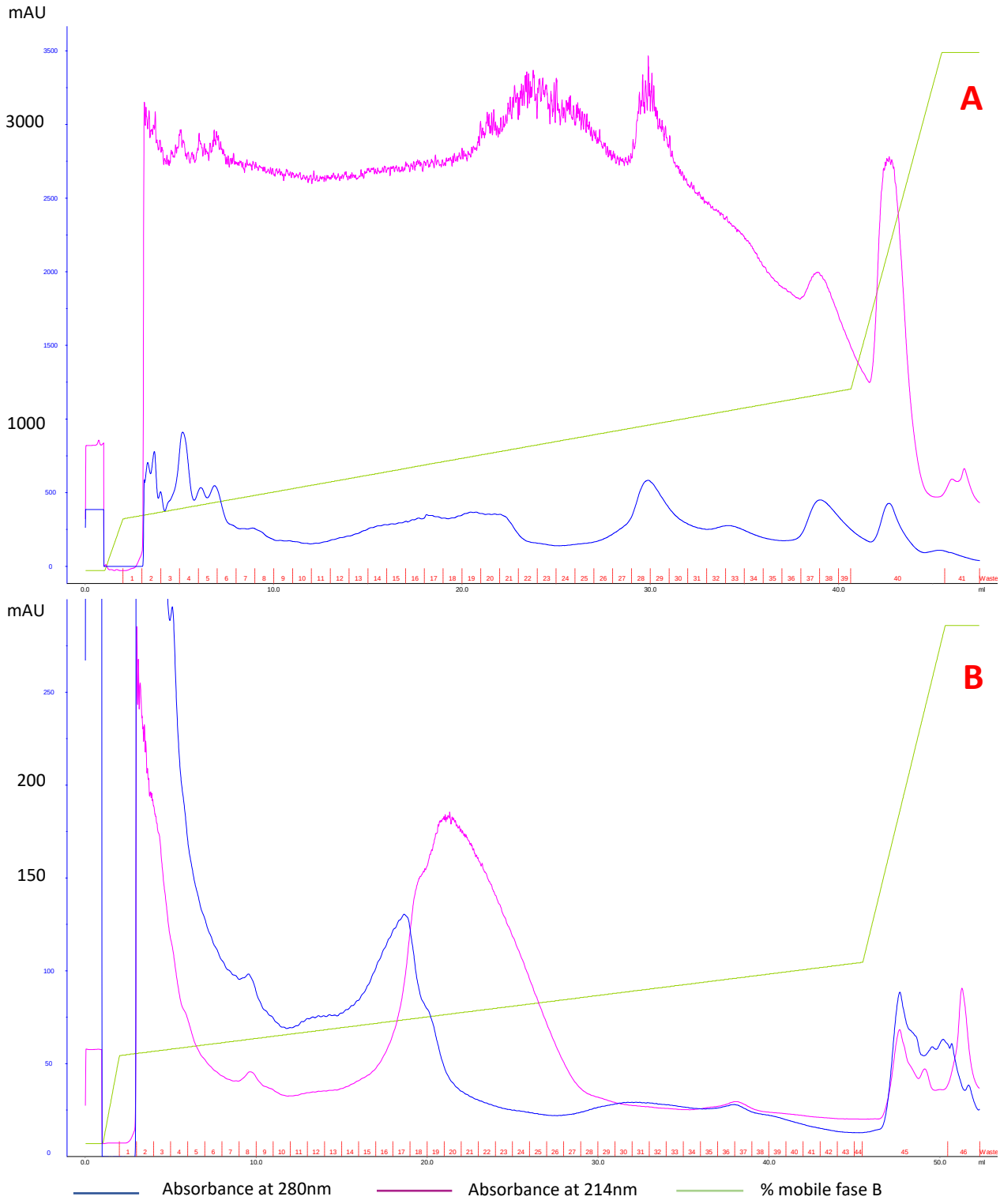

Figure 3-8 *ÄKTA reverse phase chromatography spectra. A) Reverse phase spectrum for the strain grown in BHI broth. B) Reverse phase spectrum for the strain grown in MRS broth*

3.9 Characterization of bacteriocin by mass spectrometry

Characterization of the bacteriocin by molecular mass was done by mass spectrometry. The mass spectra obtained from the mass spectrometry (figure 3-9) showed that the purified bacteriocin indeed was nisin z. This is proven by the main peak having a mass of 3329

Dalton, which is equal to the theoretical molecular weight of nisin Z. A smaller peak with the mass of 3373 Daltons is observed located near the main peak in both specters. Other small peaks could also be detected, but not in notable amounts to be important.

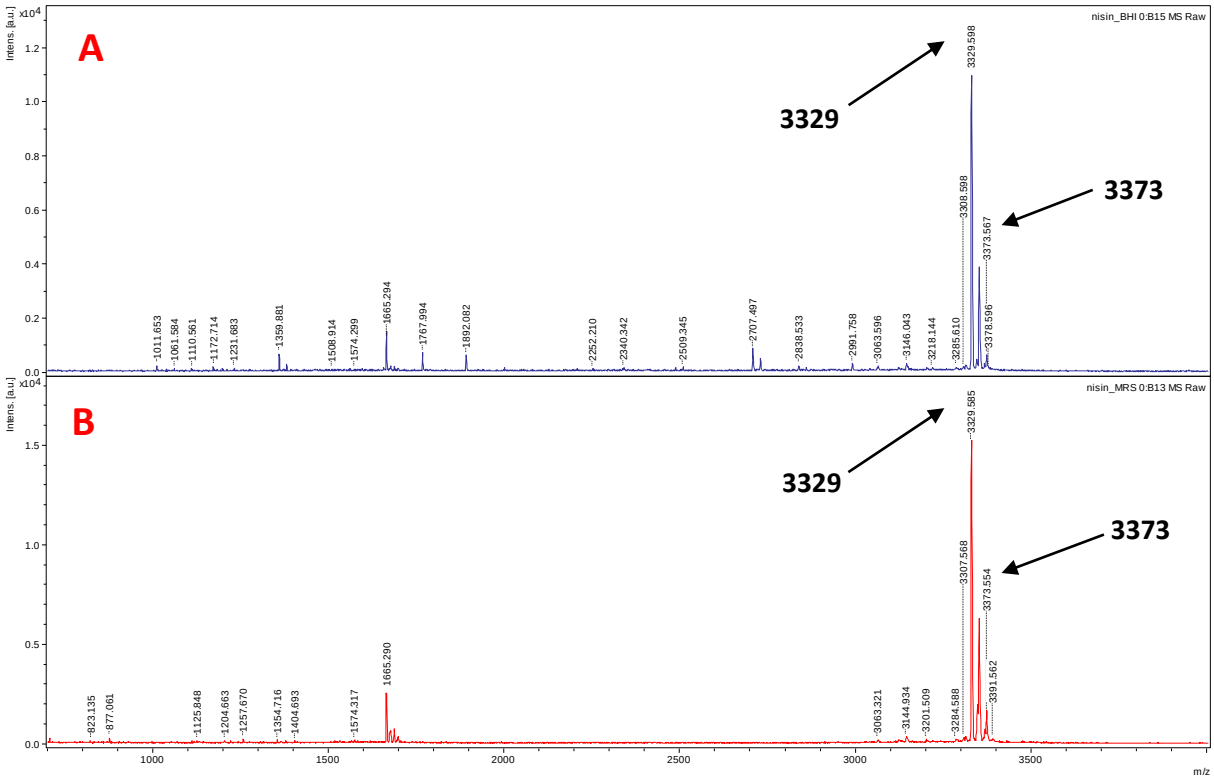

Figure 3-9 *Mass spectra of the purified nisin from BHI(A) and from MRS (B)*

3.10 Cultivating the nisin producer and S. agalactiae in water

S. agalactiae and strain 35 (*L. lactis*) were inoculated in bottles of water to see if one of the isolated nisin producers were able to inhibit growth of *S. agalactiae* in water. Dilutions of aliquots obtained from the experiment were prepared and plated as described in section 2.10. On the Edwards medium, S. agalactiae was observed growing as dark blue colonies with a zone of hemolysis surrounding the colony (figure 3-10). *L.lactis* had problem growing on the

Edwards medium, and plates of MRS agar plates were used to plate samples from the bottles where *L. lactis* were inoculated (bottle A and C from table 2-10)

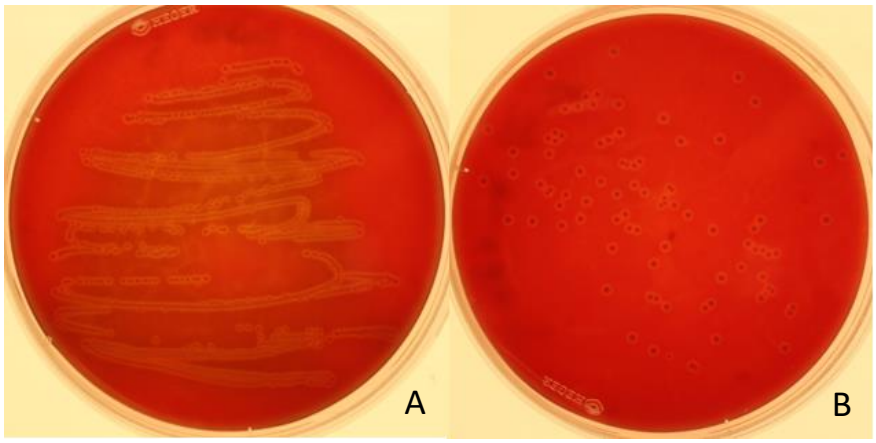

Figure 3-10 *S. agalactiae grown on Edwards medium. A: ON culture spread with loop. B : 10^-4 dilution*

Colonies were counted and used to calculate CFU/ml in the bottles at the different times of plating (Table 3-6). *L. lactis* and *S. agalactiae* were able to grow in the water for at least 48 hours but the CFU was higher for *L. lactis* than for *S. agalactiae*. Just after inoculation, we observed a slight reduction in *S. agalactiae* cells from the bottle added both bacteria, compared to cells counted from the bottle only inoculated with *S. agalactiae*. No growth could be detected on the Edwards medium plates after 24 hours, but a substantial growth was observed on the MRS plates. This indicated that strain 35 could inhibit the growth of *S. agalactiae* when inoculated in water.

Table 3-6 *Calculations of CFU/ml in the bottles.*

	0 h		24 h		48 h	
Bottle ID	**Edwards medium**	**MRS**	**Edwards medium**	**MRS**	**Edwards medium**	**MRS**
A	Not detected	not plated	not detected	$2.46 * 10^7$	not detected	$5.2 * 10^6$
B	$1.2 * 10^5$	not plated	$3.0 * 10^6$	not plated	$6.0 * 10^5$	not plated
C	$8.0 * 10^4$	not plated	not detected	$1.16 * 10^7$	not detected	$2.8 * 10^6$
D	Not detected	not plated	not deteced	not detected	not detected	not detected

Bottle A: inoculated with L. lactis, Bottle B: inoculated with S. agalactiae, Bottle C: inoculated with L. lactis and S. agalactiae, Bottle D: plain water

4 Discussion

4.1 Screening

In this study, bacteria producing bacteriocins against the two fish pathogens *S. agalactiae* and *Y. ruckeri* were obtained by screening samples made from fermented fruits and vegetables against the two pathogens. We chose to use a screening protocol selective for LAB. LAB bacteria were preferred because most of them are considered GRAS, which makes them easier to make use of in the industry. The screening was based on dilutions of the samples in MRS soft agar, which were poured on agar plates. A top layer of indicator bacteria was added and sample bacteria which inhibited the growth of the indicator bacteria were picked. This protocol was preferred over a screening method based on streaking of the samples, due to results from a former master thesis done at LMG (Haldorsen, 2017). She found that this method of screening resulted in better separation of colonies compared to the streaking method.

When screening with this protocol, it is preferable with dilutions containing a cell number low enough to make it possible to see which cell is causing which inhibition zone, but at the same time has a cell number high enough to be representable for the variety in bacteria found in the sample. Because of this a protocol for making different dilutions was used. These dilutions were used by Haldorsen (2017) when screening the same samples as were used in this study (2. Sample collection) and was shown to give at least one plate with a preferable number of colonies for almost all samples. When screening the first batch of samples we had to drop the first dilution, due to low growth rate of almost all the samples.

First, we screened a collection of samples containing mainly Norwegian vegetables. These samples had, in general, a low rate of bacterial growth. The reason for the low rate of growth in these is not known, but one explanation could be non-optimal growth conditions during fermentation which would lead to a low number of bacteria in the samples. No bacteria with antimicrobial activity were found against either of the fish pathogens while screening these samples. A second collection, containing mainly exotic, foreign fruits and vegetables, was then screened. These samples had been screened before by former master students, thus we knew they were containing a preferable number of bacteria.

After the screening, we had obtained 14 antimicrobial active colonies from eight different samples against *S. agalactiae*, but none against *Y. ruckeri*. Most of the colonies were found in samples originating from exotic fruits: avocado, purple aubergine, aristo, eddo, large chili, red onion and dragon fruit. The former screening of the same samples (Haldorsen, 2017) also found bacteria from the samples made of Avocado, purple aubergine and dragon fruit (both with and without added salt). All of these bacteria were found to be producing nisin, which could be indicating that we had also found bacteria producing nisin.

In the second sample collection, the samples were made both with and without added salt. This was done because it is thought that small concentrations (1 – 2,5%) of salt could optimize growth conditions for LAB (Chikthimmah et al, 2001), thus optimizing growth for different kinds of bacteria. It could look like this had an effect. The samples made from dragonfruit (48 and 48+) were the only samples where antimicrobial active bacteria were obtained from both the sample with and without added salt. Dragonfruit was also the only source from which we found antimicrobial active bacteria in the sample added salt. A possibility could be that these variations could come from variation of variation in microbiota in different parts of the fruits and vegetables used for making samples.

No bacteria with antimicrobial activity against *Y. ruckeri* were found in any of the screened samples. It was presumed from before start that it would more difficult finding bacteriocins against gram negative bacteria, such as *Y.ruckeri*, than for gram positive bacteria like *S. agalactiae*. This is because most bacteriocins produced by gram positive bacteria work by attacking its targets cell membrane, which for Gram negative bacteria is protected by an outer membrane (Abee et al., 1995). In order to find bacteriocins active against *Y. ruckeri*, we could have screened even more samples. This could be more of samples gathered from fruits and vegetables, in addition to samples gathered from different sources such as fermented meat, dairy products or water samples gathered from different ponds and fish farms. The screening could additionally be executed in a more generic way, not optimizing growth conditions for LAB. We chose not to do so, because LAB would be easier to implement in the aquaculture industry. We decided to not look further for bacteriocin producing bacteria against *Y. ruckeri* in this study.

Spot-on-lawn inhibition assays were used to confirm the antimicrobial activity of the pure cultures against *S. agalactiae*. Untreated supernatants were used for confirming antimicrobial activity, heat-treated supernatants were used to check if the produced antimicrobials were heat-stable and proteinase K-treated supernatants were used to see if the antimicrobial agents

were proteinaceous. Three colonies were discarded from further work due to not showing any antimicrobial activity against *S. agalactiae* in either the plain or heat-treated supernatant.

For some of the strains, single colonies were observed growing in the inhibition zone. This could indicate the development of resistance in *S. agalactiae*. Prior observations have been made of sensitive strains developing resistance against nisin (Gravesen et al., 2004). At this point it is difficult to say if these observations are due to resistance, or if it is just a result of the indicator overgrowing the amount of bacteriocins available in the drop of supernatant.

4.2 Characterization of the bacteriocin

We wanted to further investigate if the molecules causing the antimicrobial activity were bacteriocins, and which bacteriocins they were. By sanger sequencing of the 16S rRNA gene, we concluded that all the isolated strains were *L. lactis*. We had also observed that the bacteria were unable to inhibit the growth of a nisin producer. Because bacteriocin producers are immune to their own produced bacteriocins (Koponen et al., 2004), we wanted to see if the strains could also be producers of nisin. Based on the former observations, we did PCR and sanger sequencing of the nisin gene, which told us that all strains had the gene encoding a variant of nisin, nisin Z(A-1). Two of the strains had some differences from what is found in the sequence for the nisin Z gene. The sequence of strain 6a was missing the three last amino acid in the sequence. This is thought to be a result of poor quality of DNA amplified during the PCR. Strain 8 contained a proline instead of a serine. Because this difference is located in the propeptide region of the sequence (uniport, 2018), it is expected to not have an effect.

Interestingly, the inhibition spectra found for our isolated strains when testing against 46 different strains varied some from the inhibition spectrum found for a nisin producer tested against the same 46 indicator bacteria (Table 3-2). Most of the isolated strains inhibited the growth of the three strains of *Entereococcus facecalis* (158B, 111A, 29C), one of the *Lactococcus lactis* strains and *Leuconostoc gellidium* which the nisin producer did not inhibit. Our strains did on the other hand not inhibit the growth of two of the *Listeria monocytogenes* strains, *Pediococcus pentosaceus* and *Staphylococcus aureus*, which the nisin producer inhibited. We found it especially interesting that the strains did not inhibit *S. aureus.* Several studies have shown that nisin inhibits the growth of *S. aureus* quite well (Piper et al., 2009, Haldorsen , 2017). When Haldorsen (2017) screened the same samples as were screened in this study, she found several nisin producers inhibiting the growth of *S. aureus*. An explanation to this could be that we used supernatant for the inhibition assays, while cell

cultures were used in the assays made for the nisin producers and in the case of screening of these samples against *S. auerus*.

4.3 Purification of bacteriocin

When we knew that the bacteriocins produced by our producers were all nisin, we decided that we wanted to purify nisin produced by one of our strains. Before we started the purification, we wanted to find out if bacterial growth and antimicrobial activity varied between the different strains. REP-PCR of the isolated strains resulted in seven different fingerprints (figure 3-5), indicating that we had isolated 7 different strains of *L. lactis*. The strains that shared fingerprint were obtained from the same sample, indicating that bacteria found in the same samples were the same strain.

One strain representing each of the fingerprints were chosen to test for differences in growth and antimicrobial activity in different media. This was done by making growth curves, spot-on-lawn inhibition assays and antimicrobial micro titer assays. All the strains showed similar patterns in both growth and antimicrobial activity, where the strains grew best in BHI, but had more antimicrobial activity when grown in MRS. Based on the antimicrobial micro titer assay it seemed like the strain showing the most antimicrobial activity was the strain 35.

MRS has been shown to be one of the preferred growth mediums for many LAB, promoting abundant growth and also a relatively high bacteriocin level (Garsa et al., 2013). Interestingly we saw that our strains were growing at a higher rate in BHI than in MRS, but showed a higher antimicrobial activity in when grown in MRS. Because of this, we decided to purify nisin from one culture of strain 35 grown in each of the media, to see which medium we were able to retrieve the most bacteriocins from.

We managed to retrieve twice the amount of bacteriocins from the culture grown in BHI than from the culture grown in MRS. Whether these results are representable for the actual bacteriocins present in the culture or not, is difficult to say. The calculated BU for the purification of culture grown in MRS indicates that only 30% of the BU calculated in the initial supernatant, were retrieved after the reverse phase chromatography. It is not possible to say if the remaining 70% was caused by other antimicrobial agents produced when grown in MRS, or if these 70% have been lost during the purification by manners we have not discovered.

Research has shown that purification methods involving several steps, including precipitation and chromatography, often ends up with high purity but relatively low yields (Garsa et al.,

2013, Meghrous et al., 1997). It can thus be proposed that this method for retrieving bacteriocins is not suited to compare yield obtained from different broths.

Another inaccuracy with the method used, could come from the way the amount of bacteriocin were calculated. When testing by two-fold dilutions in a micro titer plate, the calculated BU are doubling for each well of growth inhibition. This leads to very strict numbers when calculating BU/ml, with big gaps between each well inhibited in the later wells. One well of inhibition difference could come from inaccuracies during tittering, differences in growth and concentration of indicator ON culture and slight differences in incubation time between different prepared plates.

Mass spectrometry showed that the purified bacteriocin had a mass of 3329 Dalton, matching the molecular weight of nisin Z. The mass spectra did in addition show that the nisin was quite pure in both rounds of purification. A peak of 3373 Daltons was observed next to the identified nisin z. The differences of these peaks are 44 Daltons, probably a result of posttranslational modifications of the bacteriocin. Also, it can be seen from these spectra that other small peaks are present. These peaks are not abundant enough to need further discussion. We can thus conclude that the substance showing antimicrobial activity against *S. agalactiae* was indeed Nisin Z.

4.4 Challenging nisin Z producer *L. lactis 35* against Fish pathogen *S. agalactiae* in water

Protein purification is a comprehensive and expensive process (Snyder and Worobo, 2014). Because of this, it would be more time efficient and economically beneficial to use a nisin producing *L. lactis* directly to the fish as a probiotic, than to use purified nisin. To explore the possibility of using *L. lactis* as a probiotic for controlling outbreaks of *S. agalactiae*, we cultivated the two bacteria in bottles containing yeast peptone-water. We saw that both bacteria managed to grow in the water separately for at least 48 hours, but that *L. lact*is was dominant in the water when the two bacteria were mixed in the same water container.

Agar plates made of modified Edwards medium were used to identify the presence of *S. agalactiae*. We observed that *L. lactis* had problems with growing on Edwards medium. Because of this we used MRS plates to observe all bacteria growth, and Edwards medium to observe growth of *S. agalactiae*. Because no colonies from bottle C were detected on the plates of Edwards medium after 24 hours of growth, the assumption was made that the growth on the MRS plates from bottle C corresponded to amount of *L. lactis* in the bottle. Because

the least diluted concentration plated was a 10^{-2} dilution, it could be possible that S. agalactiae could be detected by plating the water without making dilutions. This would still make the CFU/ml to less than 100 for *S. agalactiae*. Compared to the $1{,}16*10^{7}$ CFU/ml calculated of *L. lactis* at the same time, this is not a huge amount. By these results we have shown that *L. lactis* was able to restrict the growth of S. agalactiae when inoculated together in water.

Research has shown that *L. lactis* could have positive effects when added as a probiotic to fish (Balcázar et al., 2007). To find out that nisin Z is inhibiting the growth of *S. agalactiae* was a quite nice result. This is mostly because of nisin already being a commercialized bacteriocin which has been used in food industry for a long time(Snyder and Worobo, 2014). This makes it much easier to introduce the bacteriocin in fish farms for controlling outbreaks of *S. agalactiae*. Nisin could be applied to aquaculture either as a food additive or added directly to the water. This could be done, either in the form of purified nisin or as a probiotic by adding the producer. Because of *L. lactis* being a GRAS-organism, the process of implementing the bacteria in fish farms would be a relatively easy process. However, this was just a preliminary test, and more experiments have to be done before adding a nisin producing *L. lactis* to fish farms for controlling outbreaks of *S. agalactiae*.

Conclusion and future work

In this study, we showed that it is possible to find bacteriocinogenic LAB from fermented fruit and vegetables against *S. agalactiae* but not against *Y. ruckeri*. All the bacteriocinogenic bacteria were identified as *L. lactis*. The produced bacteriocins were all identified as the lantibiotic nisin Z.

Nisin Z was successfully purified from *L. lactis* strains 35 through a standard purification process involving protein precipitation and chromatography. The same strain of *L. lactis* was also observed being able to inhibit *S. agalactiae* when inoculated together in water.

Further work should be done containing more research around testing the use of nisin Z for controlling outbreaks of *S. agalactiae* in fish farms. Both purified nisin and nisin producing *L. lactis* as a probiotic could be used. Because nisin purification is expensive and time consuming the focus should be on exploring the use of *L. lactis* as s probiotic in fish. This research should be focused on finding out if growth conditions found in fish farms and fish are optimal for the producer, in addition to exploring the consequences an addition of this bacterium could have to the existing microbiota in the fish farms and in the fish itself. Also, it could be an idea to further research if *S. agalactiae* is developing resistance against the nisin.

It could also be made new efforts for finding bacteriocin producers against *Y. ruckeri*. It would be recommended to use samples made from other sources than fermented fruits and vegetables, like fermented meat products, dairy products or samples from fish tissues or water samples obtained from the fish farms where the bacteriocins are intended for use.

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Apendix:

Figure A-1: Multiple alignment of the aminoacid sequeneces gotten from sanger sequencing of the nisin- gene for all the strains. The two first sequences are the sequences for Nisin A and Nisin Z, found in the NCBI database.

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