Norwegian University of Life Sciences

Master's Thesis 2018 60 ECTS

Faculty of Chemistry, Biotechnology and Food Science

Characterization of Lactic Acid Bacteria from infants gut with antimicrobial activity against *Lactobacillus rhamnosus GG*

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Biotechnology – Molecular Biology

Acknowledgements

The work presented in this master thesis was performed at the Faculty of Chemistry, Biotechnology and Food Science at the Norwegian University of Life Science, with Professor Knut Rudi and Professor Dzung Bao Diep as supervisors.

First, I want to thank my supervisors for introducing me to this project and for giving me the opportunity to participate in this collaboration. Thanks for clever ideas and solutions to any problem. Both of you have been positive, enthusiastic and inspiring in your guidance, and for that, you have my deepest gratitude.

I would also like to thank Kirill Ovchinnikov for sharing your knowledge and inspiring thoughts about bacteriocins. Further, I would like to thank Amar Telke, Jane Ludvigsen and Ekaterina Avershina for all your help with statistical and bioinformatic tasks. Also, I would like to give a special thanks to Inga Leena Angell for all the help at the Microbial Diversity Lab, and Juan José Jiménes Martínes for inspiring guidance through the writing process.

I am very grateful for all the members of Laboratory of Microbial Gene Technology, Microbial Diversity Lab, ACD Pharma and all the Master students, for creating a great working environment.

Finally, thanks to all my friends and family, for always believing in me and supporting me. And a special thanks to my husband Patrick - your patience and support have meant a lot to me!

Ås, September 2018

Amanda Morken Andersen

Abstract

There are major individual differences in how the probiotic strain *Lactobacillus rhamnosus GG* (LGG) colonizes the intestine of infants. Understanding these differences will be of great importance in relation to the use of probiotics. A potential explanation could be competition from other lactic acid bacteria in the intestines. Therefore, the aim of this master thesis was to identify mechanisms for competition between LGG and other lactic acid bacteria.

The study material included a subset of fecal samples from the Prevention of Allergy among Children in Trondheim (ProPACT) cohort. Inhibition of LGG was detected trough cultivation and screening for antimicrobial activity, and the bacteria with antimicrobial activity was isolated and identified as *E. faecalis*. Furthermore, several putative bacteriocins were detected in the bacterial genomes. Therefore, it is believed that the antimicrobial activity against LGG might be caused by bacteriocin production from *E. faecalis*. However, a correlation analysis based on a quantitative PCR screening of LGG and *Enterococcus* revealed a positive correlation of approximately 50 %. This may indicate a form of dependency between these two variables and not only competition.

In conclusion, the results presented in current study improved our understanding of potential inhibition mechanisms and supports the hypothesis that the effect of probiotics can be dependent on the intrinsic microbiota.

Sammendrag

Det er store individuelle forskjeller i hvordan den probiotiske stammen *Lactobacillus rhamnosus GG* (LGG) koloniserer tarmen til spedbarn. Det å forstå disse forskjellene vil ha stor betydning i forhold til bruken av probiotika. En potensiell forklaring kan være konkurranse fra andre melkesyrebakterier i tarmene. Derfor var målet med denne masteroppgaven å identifisere mekanismer for konkurranse mellom LGG og andre melkesyrebakterier.

Studiematerialet inkluderte et utvalg av fekalprøver fra studien Prevention of Allergy among Children in Trondheim (ProPACT). Inhibering av LGG ble påvist gjennom dyrkning og skreening for antimikrobiell aktivitet og bakteriene med antimikrobiell aktivitet ble isolert og identifisert som *E. faecalis*. Videre ble det påvist flere potensielle bakteriosiner i de bakterielle genomene. Det antas derfor at den antimikrobielle aktiviteten mot LGG kan være forårsaket av bakteriosinproduksjon fra *E. faecalis*. En korrelasjonsanalyse basert på en kvantitativ PCR-skreening av LGG og *Enterococcus* viste imidlertid en positiv korrelasjon på om lag 50 %. Dette kan indikere en form for avhengighet mellom disse to variablene, og ikke bare konkurranse.

Det kan konkluderes med at resultatene presentert i denne studien bedret vår forståelse av potensielle inhiberingsmekanismer. I tillegg støtter resultatene oppunder hypotesen om at effekten av probiotika kan være avhengig av den iboende mikrobiotaen.

Abbreviations

AD	-	Atopic Dermatitis
AMPs	-	Antimicrobial peptides
BHI	-	Brain Heart Infusion broth
BLAST	-	Basic local alignment search tool
DNA	-	Deoxyribonucleic acid
dNTPs	-	Deoxynucleotide triphosphates
dsDNA	-	Double stranded DNA
EntA	-	Enterolysin A
EJ97	-	Enterocin EJ97
G-/G+	-	Gram negative/Gram positive
GI	-	Gastrointestinal
gDNA	-	Genomic DNA
LAB	-	Lactic acid bacteria
LGG	-	*Lactobacillus rhamnosus GG*
mRNA	-	Messenger ribonucleic acid
MRS	-	De Man Rogosa and Sharpe broth
NGS	-	Next generation sequencing
o/n	-	Over night
OTU	-	Operational Taxonomic Unit
PCR	-	Polymerase chain reaction
ProPACT	-	Prevention of allergy among children in Trondheim
qPCR	-	Quantitative PCR
RiPPs	-	Ribosomal synthesized and post-translationally modified peptides
RNA	-	Ribonucleic acid
rRNA	-	Ribosomal ribonucleic acid
UN	-	United Nations
WHO	-	World Health Organization

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1. Introduction

The number of microbial species with probiotic properties is impressive. However, when it comes to colonization of different probiotic strains in the intestine major individual differences is observed. Understanding these differences will be of great importance in relation to the use of probiotics. A possible explanation could be competition from other lactic acid bacteria in the intestine due to limited space or nutrition. To maintain the bacterial population many bacteria produces antimicrobial substances termed bacteriocins which can reduce the numbers of competitors. Bacteriocin-producing bacteria are found in all environments, included the gut microbiota.

1.1 Gut microbiota

The human gastrointestinal (GI) tract represents one of the largest interfaces between the host and environmental factors (Thursby and Juge, 2017). In an average lifetime, tons of food along with microorganisms from the environment pass through the human GI tract. The collection of microorganisms in the GI tract is termed as the gut microbiota. It is a complex and a dynamic system with a great diversity. Besides, it varies between individuals and can change over time, especially at early development and in the case of illness (Catherine et al. 2012).

1.1.2 Gut microbiota and human health

The understanding of how the microbiota in the gut influences human health is of great interest. Most of the microbes in the intestine are harmless and beneficial to the host. They protect against pathogen microorganisms and contributes to normal maintenance of the immune system (Catherine et.al. 2012). Different factors like genetics, birth deliver, and diet can influence the microbial colonization. Development of the gut microbiota in early childhood is especially important and will have a great influence on the human health during the life time (Rodriguez et al. 2015). As mentioned, the microbiota offers many benefits to the host. However, these mechanisms can be disrupted because of an altered microbial composition, known as dysbiosis. Dysbiosis can be defined as changes in the intestinal flora, and several factors like lifestyle, diet and medications can cause this condition. There is an increasing evidence that dysbiosis of the gut microbiota is associated with several intestinal disorders such as allergy, asthma, obesity, irritable bowel syndrome, inflammatory bowel diseases and coeliac disease (Carding et al. 2015). It is believed that the interaction between the gut microbiota and the immune system may be a major influencing factor, and that the

development can be prevented or delayed by modification of the gut microbiota (Groele et al. 2017). Today, probiotics represent the most common supplement used during intestinal dysbiosis (Banna et al. 2017).

1.1.3 Probiotics

According to the WHO and the Food and Agriculture Organization of the UN, Probiotics are defined as live microorganisms that are believed to provide health benefits when consumed in adequate amounts (WHO, 2002). The number of microbial species and genera which may have probiotic properties is impressive. However, only strains classified as lactic acid bacteria (LAB) are the most important regarding to food and nutrition (Kechagia et al. 2013). The group lactic acid bacteria are comprised of a wide range of G+, usually non-motile, non-sporing rods and cocci, that utilize carbohydrates fermentatively and form lactic acid as end product (Aguirre et al. 1993). *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Enterococcus* are all members of this group, where *Lactobacillus* is the largest genus with about 100 species (Sherwood et al. 2014). LAB are widespread in nature and often common in milk and dairy products, intestinal tracts and mucous membranes of mammals. They are useful microorganisms in foods because they are capable of inhibition of foodborne bacteria. The preservative effect of LAB is primarily due to the homolactic fermentation of sugar, which results in large amounts of lactic acid that lowering the pH (Eijsink et al. 2002). LAB also display various surface determinants that are involved in their interaction with mucus and intestinal epithelial cells (Bermudez-Brito et.al. 2012).

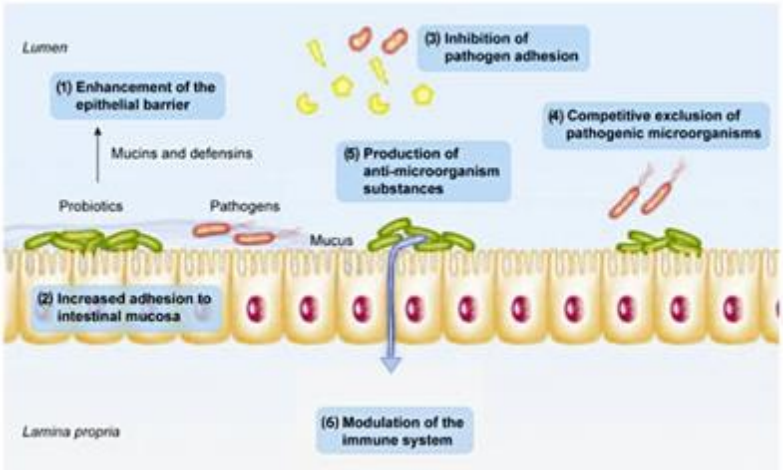

Figure 1-1 Major mechanisms of actions of probiotics; Enhancement of the epithelial barrier (1), Adhesion to intestinal mucosa (2), Competitive exclusion and inhibition of pathogens (3 and 4) and Production of Antimicrobial Substances (5) which can result in strengthening of the gut epithelial barrier and immunomodulation (6) (Bermudez-Brito et.al.,2012).

Adhesion of probiotics to the intestinal mucosa is one mechanism of action of probiotics that are important regarded the interaction between probiotic strains and the host (Fig. 1-1). It also plays a crucial role when it comes to competitive exclusion of pathogens, and the modulation of the immune system. The interaction between probiotics and mucus can induce the release of small peptides from epithelial cells termed defensins. These peptides are active against bacteria, viruses and fungi, and contribute to strengthening of the gut epithelial barrier (Bermudez-Brito et al. 2012). Moreover, different mechanisms and properties of probiotics can result in competitive exclusion of pathogens. In general, probiotic strains are able to inhibit the attachment of pathogenic bacteria by means of steric hindrance at intestinal epithelial cell binding sites (Kechagia et al. 2013). The competition for available nutrients can also result in environmental modifications. Bacteria can modify their environment to make it less suitable for competitors by production of antimicrobial substances, such as lactic acid or bacteriocins. Lactic acid has a strong inhibitory effect against Gram-negative (G-) bacteria and have been considered as the main antimicrobial compound responsible for probiotic inhibition of pathogens (Bermudez-Brito et al. 2012).

When it comes to probiotics and human health, several trials have shown that probiotics may modulate the intestinal microbiota and be important through immunomodulation. The probiotic interaction with epithelial and dendritic cells and with macrophages and lymphocytes can exert an immunomodulatory effect (Meneghin et al. 2012). By these interactions probiotics may have a considerable potential for therapeutic or preventative applications for several gastrointestinal disorders.

1.1.4 Prevention of Allergy among Children in Trondheim (ProPACT)

Allergic diseases have become a major public health problem (Rodriguez et al. 2015). The Prevention of Allergy among Children in Trondheim (ProPACT) cohort aimed to investigate whether a probiotic supplement given to pregnant women could prevent allergic diseases and atopic sensitization during the offspring's first 2 years. In total, 415 pregnant women were recruited from September 2003 to September 2005 and the clinical examination were completed in December 2007 (Dotterud et al. 2010). This study was a randomized, double-blind trial where pregnant women received probiotic supplement. The probiotic milk, Biola® contained *Lactobacillus rhamnosus GG* (LGG), *Bifidobacterium animalis* subs. Lactis Bb12 and *Lactobacillus acidophilus* La-5, and was given to the mothers from pregnancy week 36 to 3 months after birth (Dotterud et al. 2010). Cord blood, venous blood and stool samples were collected for further analysis. As a result, the study showed that probiotics given to pregnant

women reduced the cumulative incidence of atopic dermatitis (AD) among the infants (Dotterud et al. 2010). AD is an allergic inflammatory disease, and changes in environmental factors are a likely driver for the dramatic increase in the prevalence the last three decades (Avershina et al. 2016).

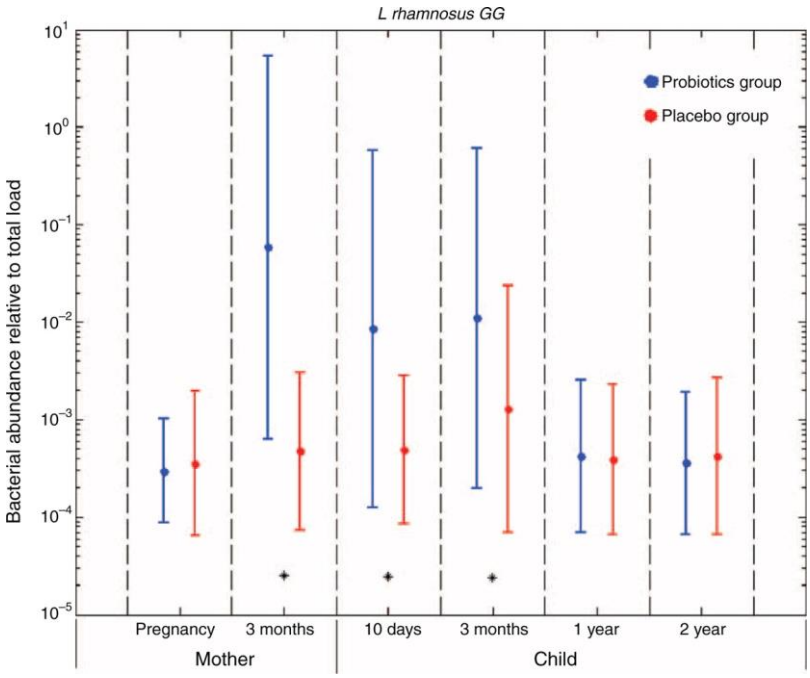

Figure 1-2 Quantitative PCR analysis showing the abundance of LGG relative to total bacterial load (Dotterud et.al.,2015).

Previous analysis of the samples from the ProPACT cohort have showed that the abundance of LGG increased in children from the probiotic group compared with those from the placebo group (Fig. 1-2). The fact that only LGG transiently colonized the children, indicating different ability of probiotics to transfer from mother to child (Dotterud et al. 2015). Based on these recent findings, a hypothesis of the probiotics is that the effect can be dependent on the intrinsic microbiota (Avershina et al. 2016).

1.1.5 *Lactobacillus rhamnosus GG*

Lactobacillus is a facultatively anaerobic, G+, nonmotile and non-spore-forming LAB. This genus contains several species found in the intestine, mouth and in the natural flora of the vagina (Sherwood et al. 2014). Ever since *Lactobacillus rhamnosus GG* (LGG) was identified as a potential probiotic strain in 1985, it has been one of the most widely studied and used probiotic strains (Segers et al. 2014). Today, LGG is successfully used in dietary supplements and foods. Due to its resistance to acid and bile, good growth characteristics and adhesion capacity to the intestinal epithelial layer, it survives trough the GI tract and can help restore and maintain the natural balance of good bacteria in the gut (Chr.Hansen. 2018).

1.2 Bacteriocins

1.2.1 Nature, function and structure of bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial peptides (AMPs) produced by numerous G+ and G- bacteria and some archea, normally targeting species or genera closely related to the producers (Umu et al. 2016). The production of bacteriocins maintains the bacterial population by reducing the numbers of competitors to obtain more space and nutrients in the surrounding environment (Yang et al. 2014). Bacteriocins differs from antibiotics in the basis of synthesis, mode of action and toxicity (Cleveland et al. 2001). Traditional antibiotics usually act as enzyme inhibitors that causes damage to protein synthesis, cell wall synthesis, DNA synthesis or other metabolic pathways. Bacteriocins on the other hand are more target-specific and are generally known to exert their activity on target cells by permeabilization of the cell membrane. This causes a leakage and loss of ion gradient leading to cell death (Diep et al. 2007). The production of bacteriocins is energy consuming and the production is therefore often regulated. The regulation is fine-tuned according to cell density and a phenomenon known as quorum sensing (Fig. 1-3).

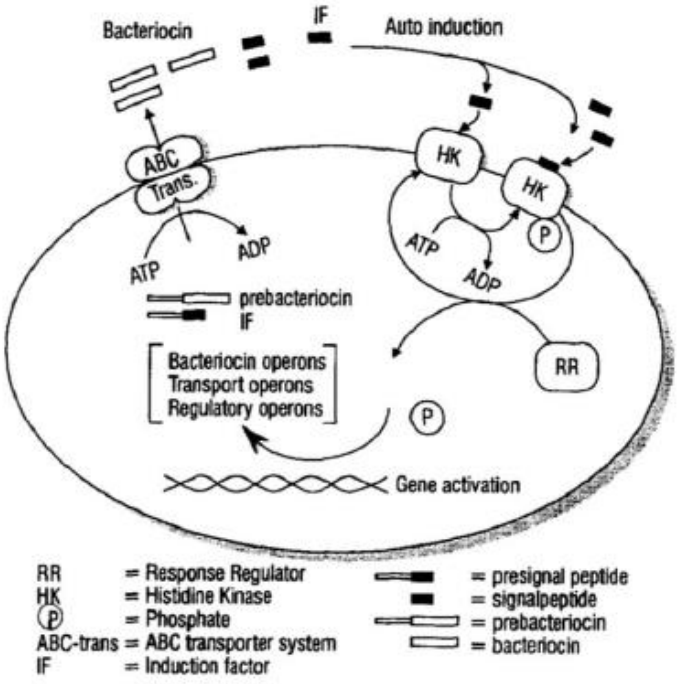

Figure 1-3 Overview of how bacteriocin production can be regulated by quorum sensing (Nes et al. 1996).

By secretion of signaling molecules called pheromones, the bacteria can sense their own growth compared to competing bacteria. By this they can communicate and coordinate the production of bacteriocin, ensuring that full bacteriocin production is initiated only when its useful for the cell (Nes et al. 1996). The signal peptide (IF) acts as a pheromone and activates

transcription of the bacteriocin genes. The genes involved in the production of active bacteriocins are usually in operon clusters, and include a structural gene, transporter gene, immunity gene and other regulatory genes (Cleveland et al. 2001). The bacteriocin producer is usually protected against its own bacteriocin by the immunity protein, and most of the bacteriocins are exported from the cell by their related ABC transporters (Nes et al. 2007).

1.2.2 Classification and practical usage of bacteriocins

Bacteriocin-producing bacteria are found in all environments, included the gut microbiota. However, bacteriocins produced by G+ bacteria are mostly LAB and are of particular interest because LAB are considered as beneficial bacteria and generally regarded as safe (GRAS) (Cleveland et al. 2001). Normally bacteriocins produced by G+ bacteria are small, heat stable antimicrobial peptides often classified into two main classes; class I (lantibiotics) and class II (non-lantibiotics). The latter can be divided further into subclasses. Class I bacteriocins containing heavily modified peptides called lantibiotics, while class II containing non-modified, or minor modified peptides (Umu et al. 2016). Recently, new ribosomally produced and post translationally modified peptides (RiPPs) with antimicrobial activity have been discovered. Therefore, a new adjusted classification of LAB, based on the biosynthesis mechanism and biological activity has been purposed (Alvarez-Sieiro et al. 2016). Fig. 1-4 illustrates the proposed classification for bacteriocins and their structure.

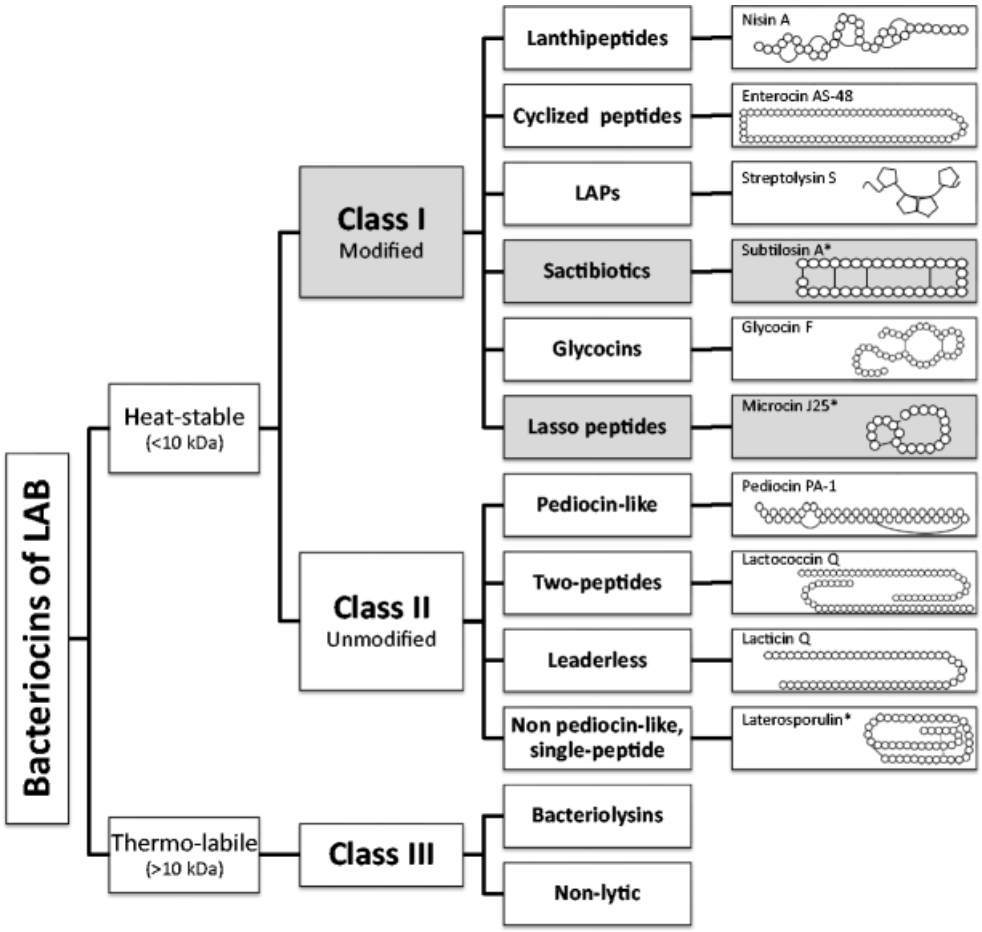

Figure 1-4 Proposed classification scheme for bacteriocins and their structures. Classes identified in silico are depicted in gray. Structure of non-lytic bacteriocins of class III still remains uncharacterized. *Bacteriocins from non-lactic acid bacteria (Alvarez-Sieiro et al. 2016).

Bacteriocins can effectively be used to control the growth of pathogens in food. The first bacteriocin that was approved as a food additive was nisin, a lantibiotic produced by *Lactococcus lactis*. This bacteriocin is currently used as a food preservative against contamination of microorganisms. Another commercially available bacteriocin is Pediocin PA-1, which inhibits the growth of *Listeria monocytogenes* in meat products (Cleveland et al. 2001). Most bacteriocins have relatively narrow inhibition spectrum, while those which have a wider spectrum and inhibit several important pathogenic species are of particular interest.

The production of bacteriocins is one of the mechanisms the bacteria use to colonize and persist in the gut (Umu et al. 2017). When it comes to the alteration of the gut microbiota and human health, a number of bacteriocins have been studied for their ability to inhibit pathogens in the gut (Umu et al. 2016). For instance, some LAB bacteriocins have been shown to inhibit pathogens such as *Staphylococcus*, *Listeria* and *Salmonella*, while other bacteriocins have been reported to inhibit multidrug-resistant enterococci (Umu et al. 2017). As a result, the production of bacteriocins may contribute to beneficial activities in the gut. However, there is

still limited information on how different bacteriocins affect the general composition of the gut microbiota (Umu et al. 2016). As a first step to obtain greater knowledge of molecular characteristics and mechanisms *in vivo*, cultivation of bacteria and screening for antimicrobial activity *in vitro* are methods that can be used.

1.3 Cultivation and screening for bacteria with antimicrobial activity

Culture techniques enables researchers to grow and stably maintain microorganisms. With basic knowledge about medium composition and physical composition that can limit or increase microbial growth, many microorganisms can be cultured from the environment. Different media can be used for the enrichment, cultivation and isolation of bacteria species of interest. With the use of selective media pathogenic and commercially useful microorganisms can easily and reliably be isolated (Kawanichi et al. 2011). Other media have no or low degree of selectivity. These media are often nutritious and well buffered to support the growth of a wide range of microorganisms. Besides the major nutritional requirements such as carbon-, nitrogen-, energy-, and nutrition sources, physiochemical factors such as temperature, presence of oxygen, pH and salinity can affect the growth.

In recent years, greater attention has been paid to antimicrobial screening. A variety of methods can be used to evaluate or screen the *in vitro* antimicrobial activity, but the most known and basic methods are the disk-diffusion and broth or agar dilution methods (Balouiri et al. 2016). In this thesis a multiple layer assay was used to screen for antimicrobial activity. This method enables screening of hundreds of bacterial colonies at the same time, where antimicrobial activity can be detected as visual inhibition zones. The steps in this assay includes cultivation, addition of an indicator, incubation, selection of colonies with visual inhibition zones and cultivation of pure cultures with antimicrobial activity for further analysis.

1.4 Polymerase chain reaction

Since the discovery of the polymerase chain reaction (PCR) in the 1980s, the technique has revolutionized the field of molecular biology. It is now a common technique used in different laboratories for a broad variety of applications. PCR is used to amplify a specific DNA region of interest. The amplified DNA is detected using gels or other fluorescence based techniques and can then be used in further applications such as cloning and DNA sequencing (Pepper et al. 2015). The components in PCR consist of a DNA polymerase, primers, deoxynucleotide triphosphates (dNTPs) and DNA template. A general PCR cycle is divided into three basic

steps; denaturation, annealing and elongation. In the first step of the PCR heat is used to separate the antiparallel DNA strands. The second step involves DNA primers, which ideally would be complementary to the ends of the DNA target. In the final step a heat stable DNA polymerase and dNTPs is used in extension of the primer, to make a complementary copy of the DNA template.

The advantages with PCR is that it enables researches to amplify a DNA target millions of times. Today thermocyclers are capable of rapidly and precisely altering and holding the different temperatures in a PCR cycle for exact time points. Many apparatuses also enable users to operate multiple temperatures on a single block, known as gradient PCR. Gradient cyclers are especially useful in the early stages of PCR assay development, and enable the user to test several temperatures to determine which temperature/primer combination that produces the best amplification (Pepper et al. 2015).

Another specialized thermocycler is quantitative PCR (qPCR), which is capable of quickly amplification and quantification by using fluorescent reporter dyes to detect the amount of newly amplified DNA. SYBR Green® and TaqMan® are the most used fluorescence detection approaches in qPCR (Pepper et al. 2015). SYBR Green involves a fluorescent dye that binds to dsDNA. This is a non-specific but cost-effective method for detecting DNA. By including a melt-curve, the specificity of the SYBR Green can be determined. During the melt-curve, the temperature is raised which resulting in denaturation of the double-stranded PCR amplicons, and comparison of the PCR amplicon melt-curve to a standard helps to verify the specificity of the amplification (Pepper et al. 2015). TaqMan assays include an internal probe which binds between the two primer-binding sites. The probe contains a fluorophore in close proximity to a quencher. Because the probe binds internally to the amplicon it results in an increased specificity. When bound to the target sequence, the probe is cleaved by the polymerase which liberates the fluorescent dye thereby enabling visualization and quantification (Pepper et al. 2015). Since internal DNA probes are designed to bind between the upstream and downstream primer-binding sites, and by this are more specific than SYBR Green, the need for melting-curve analysis is reduced.

1.4 DNA sequencing methods

The major breakthrough that forever altered DNA sequencing technology came with the development of Sanger's "Chain Termination" in 1977 (Heather et al. 2016). Sanger sequencing become the most common technology to sequence DNA years to come, and a

number of improvements the following years contributed to the development of automated DNA sequencing machines (Heather et al. 2016). Eventually, sequencers which allowed simultaneous sequencing of hundreds of samples came to be used in the Human Genome Project (Heather et al. 2016). The completion of the Human Genome Project revealed the need of greater and more advanced technologies, however high costs and limited throughput remained major barriers (Goodwin et al. 2016). The release of the first truly high-throughput sequencing platform in the mid-2000s led to second generation sequencing, referred to as the Next Generation Sequencing (NGS), which resulted in a drop in the cost of sequencing (Goodwin et al. 2016). NGS technology resulted in massively parallel sequencing with high speed and throughput and whole genomes could finally rapidly be sequenced. Sequencing by synthesis is a term used to describe numerous DNA-polymerase-dependent methods and can be classified as cyclic reversible termination (CRT) or as single-nucleotide addition (SNA). One of the most used NGS technologies is the Illumina sequencing where the sequencing is based on sequencing by synthesis using a method that detects single bases as they are incorporated into growing DNA strands. Sequencing by the Illumina platform starts with the attachment of adapters flanking the fragments to be sequenced. The adapters include sequences complementary to the flow-cell-oligos where clusters of fragments are made in a bridge like manner (Fig. 1-5). Identification of dNTPs is achieved through internal reflection fluorescence using laser channels (Goodwin et al. 2016).

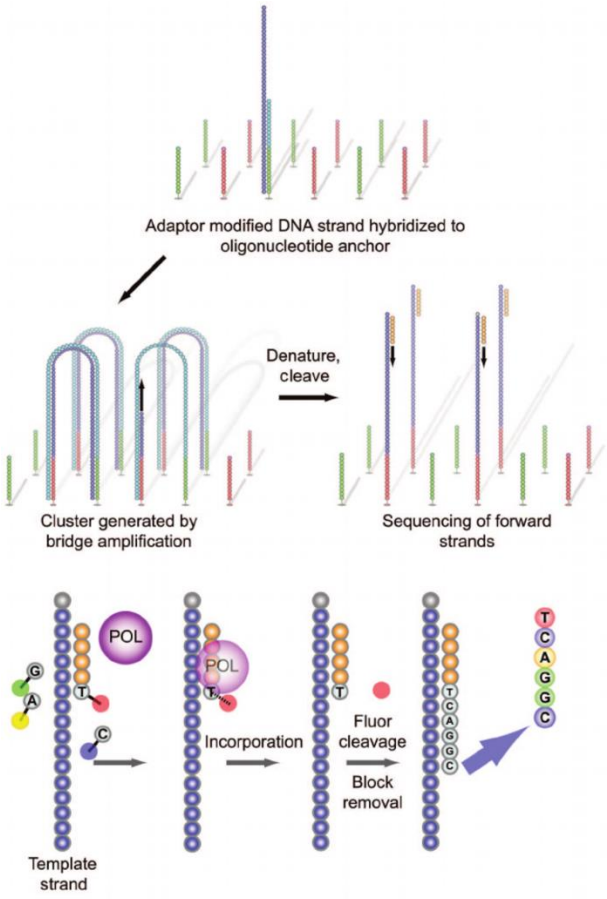

Figure 1-5 Next generation sequencing using an Illumina sequencing platform. Single stranded, adapter-modified DNA is added to the flow cell. By hybridization, bridge amplification generates clonally amplified clusters. The clusters are denaturated and cleaved, and sequencing is initiated with addition of primers, polymerase (POL) and 4 reversible dye terminators. Fluorescens is recorded and both fluor and block is removed before the next synthesis cycle (Voelkerding et al. 2009).

NGS technologies have continued to evolve. These days, a new area of sequencing is rising. Third generation sequencing differ from previous technologies by being capable of sequencing single molecules without the requirement for DNA amplification (Goodwin et al. 2016). One of the most used third generation technology methods is probably the single molecule real time (SMRT) platform (Goodwin et al. 2016). By this method DNA polymerization occurs in arrays of microfabricated nanostructures and this process can sequence single molecules in a very short amount of time (Goodwin et al. 2016). Another anticipated area for third generation sequencing is the use of nanopores for detection and quantification of chemical and biological molecules (Goodwin et al. 2016).

1.5 Bioinformatics

Bacterial genome sequences are revolutionizing the approach to identify novel genes in different bacteria (Nes et al. 2007), and the recent flood of data from genome sequences and functional genomics has given rise to new field. This field termed “Bioinformatics” combines elements of biology and computer science to analysis large datasets such as genome sequences and macromolecular structures (Luscombe et al. 2001). National Center for Biotechnology Information (NCBI) houses a series of databases and is today a leading source for public databases and software tools. With NCBIs Basic Local Alignment Search Tool (BLAST) you can easily find sequences similar to a query sequence within the same organism or in different organisms. Another bioinformatical tool is Rapid Annotation using Subsystem Technologies (Rast). This is a prokaryotic genome annotation service where metabolic reconstruction can be used to compare the functioning parts of different organisms. By listing all genes which are associated with a subsystem in the respective organism this can be used to look for similarities or dissimilarities between sequenced genomes. CLC Workbench is a tool that can be used for DNA, RNA, and protein sequence data analysis. It gives more information of the sequence of interest, such as graphics, topology, functional genes. CLC Workbench can also be used for primer and probe design like Geneious, which is another powerful tool for molecular biology and NGS analysis.

Another specified bioinformatical tool is Bagel. This is a web-based bacteriocin genome mining tool, and its unique in its ability to detect putative bacteriocin gene clusters in (new) bacterial genomes (De Jong et al. 2006). In Bagel4 you can often find the functional or structural part of the bacteriocin. These are the clusters responsible for the production of ribosomal synthesized and post-translationally modified peptides (RiPPs) and other bacteriocins (Bagel4 Webserver, 2018).

1.6 Aim of the study

There are major individual differences in how *Lactobacillus rhamnosus GG* (LGG) colonizes the intestine of infants. A better understanding of this will be of great importance in relation to the use of probiotics. A possible explanation could be competition from other lactic acid bacteria in the intestines. Based on the hypothesis that the effect of the probiotics can be dependent on the intrinsic microbiota of the infants, the main goal of this thesis was to identify possible mechanisms for competition between LGG and other lactic acid bacteria. The study material used in this study was a subset of stool samples from infants collected during the ProPACT cohort. To achieve the main goal, several sub goals was included as listed below.

1. Selection of samples from ProPACT based on a screening for OTUs with positive or negative correlation with LGG.
2. Screening and identification of bacteria with antimicrobial activity.
3. Identification of potential inhibition mechanisms.
4. Correlation analysis.

An outline of the work done in this study is illustrated in Fig. 1-6. The workflow consisted of cultivation of selected stool samples, screening for antimicrobial activity with the use of a multiple layer assay, test of inhibition spectrum by a spot-on-lawn inhibition assay, biochemical analysis including proteinase K sensitivity and heat stability, 16S rRNA gene Sanger Sequencing, Shotgun Sequencing using an illumina Miseq platform, bioinformatical analysis of the bacterial genomes, qPCR screening, and correlation analysis based on the qPCR screening.

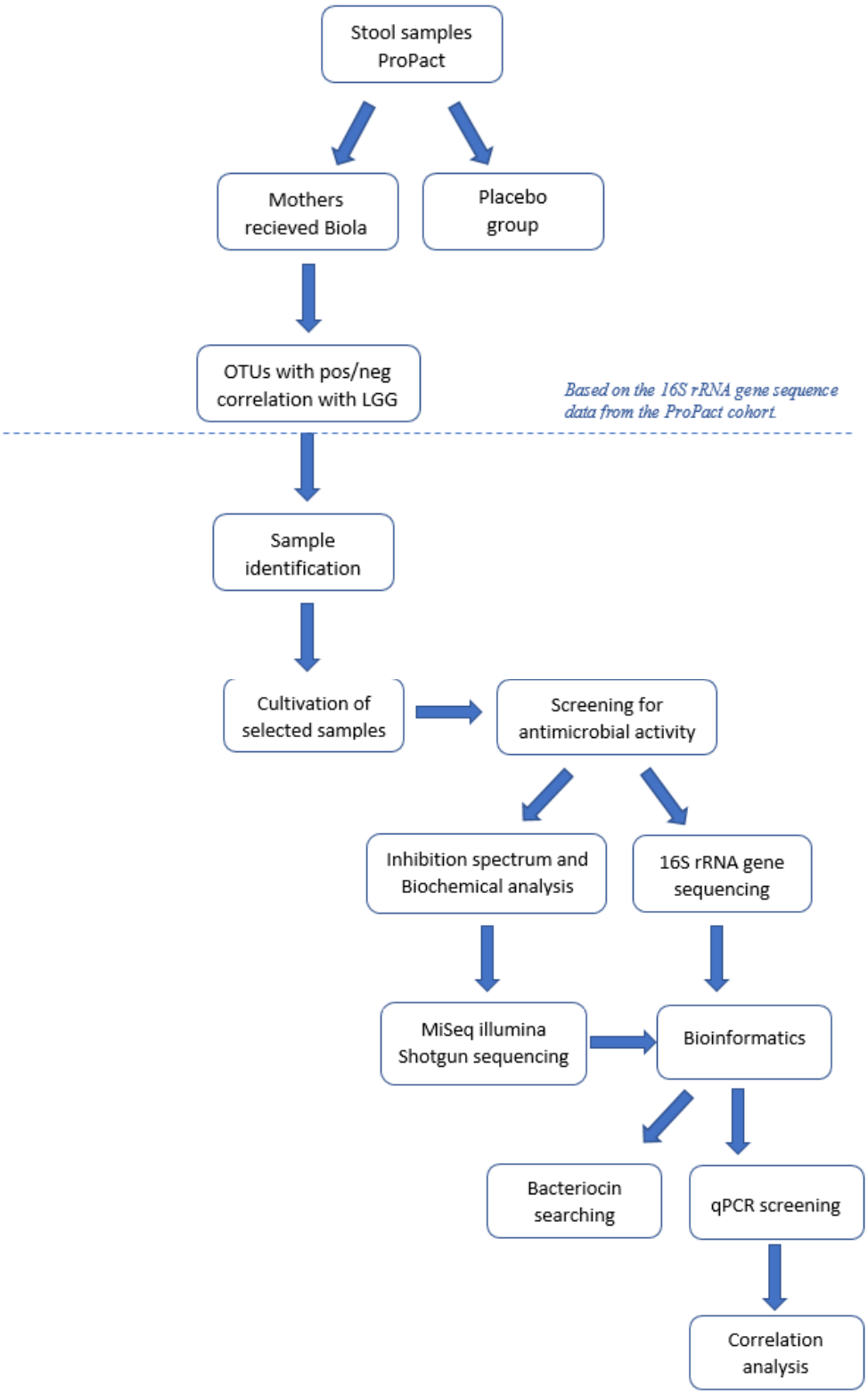

Figure 1-6 Flowchart illustrating the workflow done in this study with the main goal to identify possible mechanisms for competition between LGG and other lactic acid bacteria.

2. Materials & Methods

2.1 Collection of samples

During the Prevention of Allergy among Children in Trondheim (ProPACT) cohort, stool samples were collected from women in pregnancy week 30 to 36, and at 3 months after birth. From the infants, stool samples were collected at the age 10 days, 3 months, 1 year and 2 years. The stool samples were frozen within 2 hours after defection at -20^{o}C and delivered to the laboratory for long time storage at -80^{o}C (Dotterud et al. 2010).

The selection of samples for this current study was based on the 16S rRNA gene sequence data from the ProPACT cohort. A correlation study was performed in MatLab R2016b (Mathworks) and done by Postdoc Ekaterina Avershina at Microbial Diversity Group (MiDiv), with the aim to find OTUs with negative or positive correlation to LGG. Samples belonging to the OTUs with significant correlation to LGG were selected for further analysis. The selection of samples included only stool samples from the infants in the probiotic group. A full description of the selected samples can be found in table 3-1.

2.2 Bacterial growth media

The growth media used in this study were De Man Rogosa and Sharpe (MRS) and Brain Heart Broth (BHI) from Sigma-Aldrich. The concentrations were respectively 52 g/L and 37 g/L. Solid growth media contained 1.5 % agar powder while soft agar contained 0.8 % agar powder. The growth media were prepared following manufacture's recipe followed by autoclaving and storage at 4 ^{o}C until use. MRS medium support good growth of lactobacilli in general but has a low degree of selectivity. BHI medium were used for some of the samples to increase the growth. This medium is nutritious and well buffered and support a wide range of microorganisms. Sterile work benches with fume hood, sterile equipment and gloves were used under this study to prevent contaminations of growth media and samples.

2.3 Screening for bacteria with antimicrobial activity

Screening for bacteria with antimicrobial activity was done with the use of a multiple layer assay as illustrated in Fig. 2-1. MRS agar and MRS soft agar were used through the screening process.

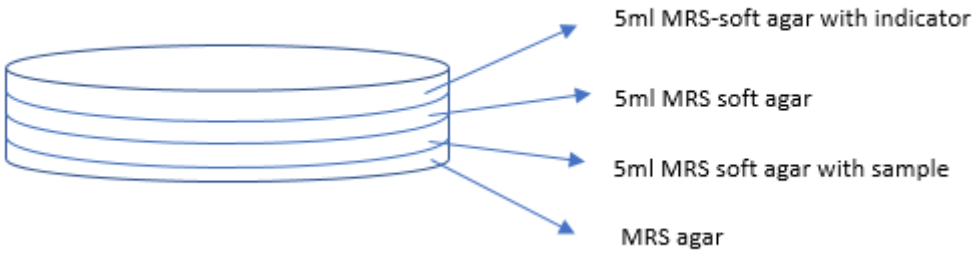

Figure 2-1 Multiple layer assay with MRS medium used in the screening for bacteria with antimicrobial activity against indicator *Lactobacillus rhamnosus GG*.

The desired number of bacterial colonies to screen for antimicrobial activity against indicator LGG, was between 500-1000 colonies per stool sample. The samples were prepared by using sterile culture tubes with 5 ml MRS soft agar. The soft agar was kept melted by placing the culture tubes in a water bath (Jumbo) with the temperature set to about 50°C. From the original stool samples 10 µl were added to a culture tube with melted MRS soft agar in the fume hood. From the first dilution 50 µl were transferred to the next culture tube and so on. The culture tubes were vortexed (Scientific Industries) between each dilution.

Some adjustments were made in the dilution series to increase the bacterial growth. Fig. 2-2 gives an illustration of how most of the samples were prepared.

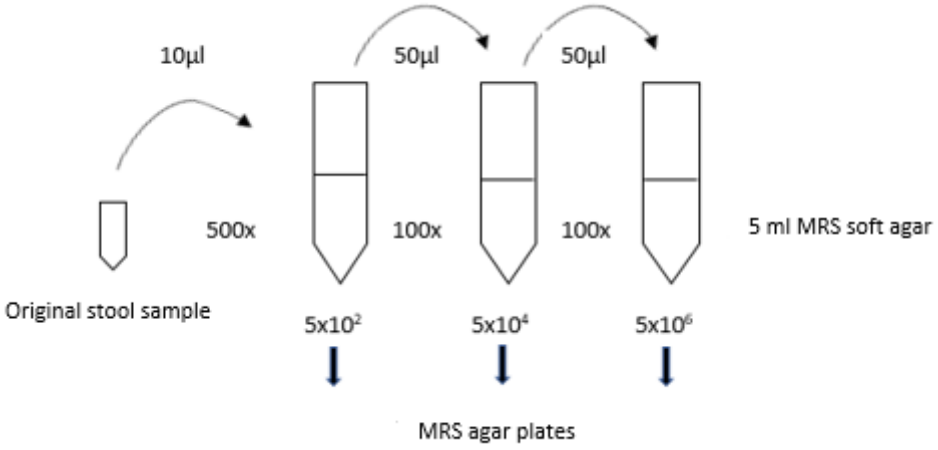

Figure 2-2 Serial dilution of stool samples in MRS medium.

Each dilution was poured onto marked MRS agar plates and the plates were solidified before 5 ml MRS soft agar was added as a middle layer. The agar plates were again allowed to

solidify before they were placed in an anaerobic growth chamber with an AnaeroGen^{TM} bag (Thermo Scientific). The plates were incubated anaerobic overnight (o/n) at 30 ^{o}C.

After incubation, 5 ml melted MRS soft agar with 25 µl o/n culture of the indicator were added to each plate. The plates were again incubated anaerobic o/n at 30 ^{o}C.

After the second incubation, colonies with inhibition zones were picked and streaked on a new MRS-plate in order to get single colonies. Afterwards, they were picked and grown as pure cultures and tested once more against the indicator and prepared for long time storage in cryo-tubes with 15 % glycerol at -80 ^{o}C.

2.4 Inhibition spectrum of antimicrobial activity

The pure cultures from the antimicrobial screening were prepared for a spot-on-lawn inhibition assay to test the inhibition ability. Fresh o/n cultures from different indicators to be tested (25 µl indicator in 5 ml melted BHI soft agar) were poured onto BHI agar plates. This medium was selected through the spot-on-lawn assay because it is a nutrient-rich medium and can therefore be used to cultivate a wide range of microorganisms. The plates solidified before 5 µl of o/n culture from the pure cultures were added on marked spots on the top of the plates. The plates were incubated under aerobic condition o/n at 30 ^{o}C. The inhibition zones were listed and scored from 0.5 to 3, where 3 gives the biggest inhibition zones. Bacterial strains with known bacteriocin production were included as controls; *Enterococcus faecium* T136, *E. faecium* L50.1, and *Lactococcus lactis* B1580. An overview of the 58 indicators that were tested is described in Appendix 2.

2.5 Proteinase K sensitivity and heat stability

Biochemical analyses were performed to study the antimicrobial activity further. The samples were concentrated by precipitation with ammonium sulfate ($(NH_4)_2SO_4$) (EMSURE® ACS,ISO,Reag. Ph Eur), heat treated and filtrated. O/n-cultures of the samples to be tested were inoculated (700 µL o/n-culture in 70 ml MRS medium) and incubated o/n at 30 ^{o}C. The next day the samples were centrifugated for 30 min at 7000 rpm (Eppendorf Centrifuge 5804 R). The pellet was discarded and aliquots (1 ml) of the supernatant was transferred to eppendorf tubes and stored at 4 ^{o}C o/n. The rest of the supernatant were transferred to a sterile Blue Cap Bottle with PipetBoy (Integra) and was 70 % saturated by adding $(NH_4)_2SO_4$. The next day the supernatant of the concentrated sample was discarded and the precipitation was dissolved in 1 ml dH_2O. Aliquots of both concentrated and non-concentrated samples were sterile filtrated with a non-pyrogenic sterile 0.20 µm filter (Sarstedt). To test heat stability,

one aliquot of the samples was transferred to an Eppendorf tube and placed at 95^{o}C (Stuart Scientific Test Tube Heater SHT1) for 5 min.

MRS agar plates with indicator LGG (25 µl indicator in 5 ml melted MRS soft agar) were made, and 10 µl of the different aliquots from the samples (fresh o/n-culture, filtrated, non-concentrated, concentrated and heat-treated) were applied to the agar plate. In addition, one more plate with the same samples was made, but on this plate 10 µl Proteinase K (20mg/ml) was added next to the marked samples. Proteinase K was also added alone to make sure that the antimicrobial activity was not due to this enzymatic solution. As a control the nisin producer *Lactococcus lactis* B1580 were included in all steps. The plates were kept in the fume hood until the plate were dry and then incubated aerobic at 30 ^{o}C o/n.

2.6 Quantification of antimicrobials in liquid

A microtiter assay was used to do a quantification of the antimicrobials produced in liquid cultures. The same samples as described in section 2.5 (fresh o/n-culture, filtrated, non-concentrated, concentrated and heat-treated), were also used in this assay. Each sample with different treatment was applied to the microtiter plate (100 µl). Growth medium (MRS) was added in each well (100 µl), and the samples were diluted in a serial two-fold manner, from row 1-10 (Fig. 2-3). This was done by pipetting carefully 5 times in each row with a multichannel pipet with a volume set to 100 µl, before the diluted samples were transferred to the next row. O/n culture of the indicator LGG were diluted (approximately 1 ml indicator per 24 ml growth medium) and 100 µl of this dilution were added in each row except row 11 (control 1). Row 12 (control 2) consisted of growth medium broth and indicator. Each well had a total volume of 200 µl. The microtiter plate were incubated at 30 ^{o}C for 4-6 hours. Some adjustments were made in the temperature and incubation time to obtain best conditions for the bacterial growth.

After incubation the plates were analyzed by using SPECTROstar Nano (BMG Labtech) with absorbance values displayed as OD (600 nm).

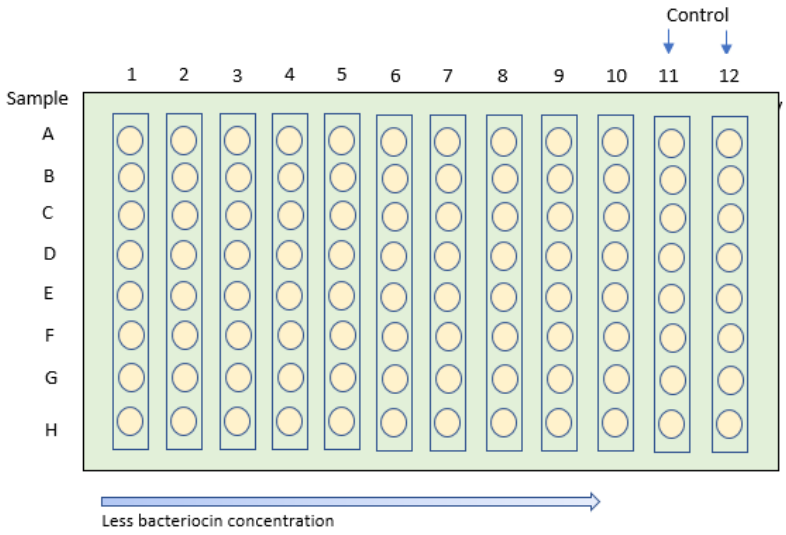

Figure 2-3 Microtiter plate used for quantification of antimicrobial activity.

2.7 Polymerase chain reaction (PCR)

2.7.1 PCR preparation 16S rRNA gene sequencing

Before the 16S rRNA gene sequencing (by GATC Biotech) a 50 µl PCR reaction was made. Each reaction contained 5x OneTaq Standard Reaction Buffer (New England BioLabs®$_{inc}$) 10 µM dNTPs (Solution Mix, New England BioLabs®$_{inc}$), 10 µM both forward and reverse primers, 1.25 units OneTaq DNA Polymerase (New England BioLabs®$_{inc}$) and 2 µl DNA (<1.000 ng). The primers used were forward primer 11F 5' TAA CAC ATG CAA GTC GAA CG 3' and reverse primer 4R 5' ACG GGC GGT GTG TRC 3' (Invitrogen by Thermo Fisher Scientific).

The PCR-run was done with a S1000^{TM} Thermal Cycler with initiation for 5 min at 94 ^{o}C and a 30x cycle of 94 ^{o}C in 45 sec, 58 ^{o}C in 1 min and 72 ^{o}C for 1,5 min. The final step was 72 ^{o}C for 5 min before the PCR-products were kept at 4 degrees until further use.

After the PCR amplification, a gel electrophoresis was performed as described in section 2.8.

2.7.2 Gradient PCR

To test the specific primers for the bacteriocin genes and to find the most optimal annealing temperature before the qPCR screening, an Eppendorf Mastercycler gradient PCR was used. The settings used consisted of 25 cycles with a gradient = 7 which had a temperature difference between 49-64 ^{o}C and default setting R = 3. Each reaction contained 1x HOT FirePol® Ready to Load, 0.2 µM both forward and reverse primers and 1 µl DNA. The primers used was EJ97 F55, EJ97 R135, EntA F586 and EntA R762 (Invitrogen by Thermo

Fisher Scientific). The primers are described in table 2-1. After the gradient PCR a gel electrophoresis was performed as described in section 2.8.

2.7.3 Quantitative PCR

Quantitative PCR (qPCR) is a precise and rapid method for nucleic acid detection. It is based on the traditional PCR, but with this method it is possible to quantify the product based on fluorescent detection. In this study two different approaches were used; First a probe based qPCR was used to do detect bacteriocin genes. Second a qPCR with the use of EvaGreen® which binds to double stranded DNA (dsDNA) was used for detection of 16S rRNA, LGG and Enterococcus. The additional melting curve analysis (HMR- high melting resolution), included in the qPCR screening of LGG, Enterococcus and 16S rRNA consisted of following; 95 ºC for 30 seconds, 60 ºC for 1 second, 70 ºC for 1 second and 95 ºC for 20 seconds.

The qPCR screening was done on the selected stool samples from ProPACT (Table 3-1), included their respective mother-child pair. In total 60 samples with extracted DNA from ProPACT was used in the screening for EJ97, EntA, LGG, 16S rRNA and Enterococcus. For a full overview of the samples used in the qPCR screening, see Appendix 1. The samples for the probe based qPCR screening was prepared and each reaction contained 1x HotFirePol Probe qPCR mix Plus, 0.2 µM both forward and reverse primers, 0.25 µM probe and 1 µl DNA. Each bacteriocin had specific primers and probes based on the sequence of the structural gene of the bacteriocins. The controls included were A1 with primers and probes for EntA, A17 with primers and probes for EJ97 and PCR-water. All the primers (Invitrogen by Thermo Fisher Scientific) used for the qPCR screening are listed in table 2-1 and the probes are described in table 2-2.

Table 2-1 Primers used in the qPCR screening.

Primer name	Sequence 5' – 3'	Target bacteriocin/bacteria	Reference
EJ97 F55 (forward)	GCA GCT AAG CTA ACG ACT TAC G	Enterocin EJ97	This work
EJ97 R135 (reverse)	TTA TGC TAC AGG GCG CTC C	Enterocin EJ97	This work
EntA F586 (forward)	GTT CGT TAC GGA TTG CGG GT	Enterolysin A	This work
EntA R762 (reverse)	AGG CAA CCA TCC GCT TTG AG	Enterolysin A	This work
Lrhamn1 (forward)	CAA TCT GAA TGA ACA GTT GTC	Lactobacillus rhamnosus	Dommels et al, 2009
Lrhamn2 (reverse)	TAT CTT GAC CAA ACT TGA CG	Lactobacillus rhamnosus	Dommels et al, 2009
EntF (forward)	CCT TAT TGT TAG TTG CCA TCA TT	Enterococcus	Collado et al., 2009
EntR (reverse)	ACT CGT TGT ACT TCC CAT TGT	Enterococcus	Collado et al., 2009
PRK341F (forward)	CCT ACG GGR BGC ASC AG	Prokaryotes	Yu et al., 2005
PRK806R (reverse)	GGA CTA CYV GGG TAT CTA AT	Prokaryotes	Yu et al., 2005

Table 2-2 probes used in the probe based qPCR screening of the bacteriocins.

Probe name	Sequence 5' – 3'	Target bacteriocin
EJ97 P93	ACA ACA ATA CGG TCG TTA TCC TTG	Enterocin EJ97
EntA P657	TGG TTT CGC AGG TTA TCG TCA	Enterolysin A

The qPCR plate was placed in a LightCycler®480 II from Roche. The program used consisted of initiation for 12 min at 95 ºC followed by a 40x cycle of denaturation for 20 sec at 95 ºC and annealing/elongation for 1 min at 60 ºC. After the run, data was downloaded and saved for further analysis. The qPCR data was imported to a quantitative PCR data analysis program (LinRegPCR) where the PCR efficiency was calculated. Excel was used to sort the data and a Pearson- and a Spearman correlation coefficient were calculated.

2.8 Gel-electrophoresis

For the 16S rRNA gene PCR-products 1 % agarose gel with PeqGreen was used to validate the PCR product. One µl Gel Loading Dye 6x Purple Loading Dye (NewEngland BioLabs$_{inc}$) were added to 5 µl of the PCR-products and mixed. The samples (5 µl) and 1kb DNA ladder (5 µl) were applied on the gel.

For the Gradient PCR products 1,5 % agarose gel was prepared and the PCR product (5 µl) was directly applied to each well in the gel. The direct application to the gel without adding loading dye is due to the 5x HOT FIREPol® used in the PCR amplification. This is a premixed solution containing all reagent required, and among the reagents there are two tracking dyes that allow to monitor progress during electrophoresis. Quick-Load® Purple 100 bp DNA Ladder (50 µg/m) from New England BioLabs®$_{inc}$ was included and applied on the gel (5 µl).

The gel electrophoresis was set to 95V for about 25 min by using PowerPac™ Basic, BioRad. The DNA from the PCR-products after the gel electrophoresis were visualized by Molecular Imager® GelDoc™ XR Imaging System.

2.9 DNA extraction and purification

Cell lysis is among the first steps for DNA extraction. Cell lysis can be achieved by disrupting cell membranes in different ways. One example is elevated temperatures, where the bacteria suspension is exposed to high temperatures that results in lysis of the cell. Another example is mechanical lysis such as bead beating. This method is commonly used for lysis of thick-walled organisms since heat, chemical and enzymatic lysis is less effective (Mao et al.,2010).

2.9.1 Preparation for 16S rRNA gene sequencing

Before the 16S rRNA gene Sanger sequencing, DNA was extracted by following the user manual from NeucleoSpin® Gel and PCR Clean-up (Macherey-Nagel GmbH & Co. KG, Germany); The mixture of 90 µl NTI and 45 µL PCR-product was added to a silica membrane column in an Eppendorf tube. To bind the DNA to the membrane the samples were centrifugated at 11000x g for 30 seconds. The silica membrane was then washed with 700 µl NT3 twice and centrifugated at 11000x g for 30 seconds. The silica membrane was dried by 1 min centrifugation at 11000x g. Elution of the DNA was done by adding 30 µL NE, the sample was left in room temperature for 1 min before centrifugation 1 min at 11000x g.

After the PCR-clean up, DNA was measured by a NanoDrop 2000 Spectrometer (Thermo Scientific). NE-buffer were used to reset the program before measuring before 2 µl of the sample was applied to the instrument. The measurement was then completed and registered.

2.9.2 Preparation shotgun sequencing

Before the shotgun sequencing the bacterial cells were prepared for DNA extraction. 1.2 ml cell culture was transferred into an Eppendorf tube and centrifugated at 13000 rpm for 5 min. The supernatant was discarded and the pellet was washed twice with 1x PBS. The pellet was desolved in 200 µl Stool Transport and Recovery buffer (S.T.A.R. buffer Roche).

Acid washed beads (Sigma-Aldrich) size of <106 µm, 425-600 µm and 2 mm (0.2 g of each type of beads) were transferred to a FastPrep tube (Starstedt) before processed twice in FastPrep 96 (MP Biomedicals) at 1800 rpm for 40 sec with 5 min rest between runs. After centrifugation at 13000 rpm in 5 min, DNA extraction was continued using Mag midi kit (LGC Genomics) following manufacturer's recommendation.

Lysis buffer BLm and protease were added to each sample and mixed before incubation at 55 ^{o}C (Biosan Thermoshaker) for 10 min. When the samples had cooled down, ethanol and fully re-suspended mag particle suspension BLm were added. The tubes were placed into contact with a magnet and when the particles formed a pellet the supernatant was removed and discarded. The magnet was moved away from the tubes and wash buffer BLm 1 was added and the pellet were resuspended. The magnet was moved into contact with the sample tubes and the supernatant was removed and discarded. This was repeated twice, second with the wash buffer BLm2. Afterwards, the pellet was placed at 55 ^{o}C (Biosan Thermoshaker) to dry before Eluation buffer BLm was added and the pellet was resuspended. The samples were incubated at 55 ^{o}C (Biosan Thermoshaker) for 10 min and vortexed periodically. The sample tubes were placed into contact with the magnet, and the eluate was removed and placed into a new sample tube.

For the measurement of the DNA concentration before illumina MiSeq shotgun sequencing and the qPCR screening, Qubit^{TM} ds DNA HS Assay Kit from Invitrogen by Thermofisher Scientific were used. The measurements were done in the Qubit^{TM} Fluoremeter.Working solution was made of 1 µl Qubit^{TM} ds DNA HS Reagent and 199 µl Qubit^{TM} dsDNA HS buffer. Two different standards were used to calibrate the instrument by adding 190 µl working solution and 10 µl of the standard. The Qubit^{TM} dsDNA HS standard #1 was 0ng/µl and Qubit^{TM} ds DNA HS standard #2 was 10ng/µl. Before measurement, 198 µl working

solution and 2 µl of the sample were vortexed and placed in the dark for 2 min. The measurement was then completed and registered.

2.11 Sequencing

2.11.1 16S rRNA gene Sanger Sequencing

To determine the DNA sequence of 16S rRNA gene from the bacteria, the samples had to be prepared according to GATC Biotech Lightrun sample requirements. Desired concentration of template DNA from purified PCR-product was between 20-80 µg/µl. The DNA template 5 µl was transferred to a 1,5 ml tube and mixed with 5 µl of the 4R primer 5 µM. The tubes were marked with barcodes and sent to GATC Biotech for High-throughput Sanger sequencing. For description of primer see section 2.7.1.

2.11.2 Illumina MiSeq Shotgun Sequencing

An illumina MiSeq Shotgun Sequencing was done with the aim to do a whole genome sequencing of the bacteria with antimicrobial activity and inhibitory effect against LGG. Nextera XT DNA Library Prep Kit (Illumina, USA) was used following the manufactures recommendations. The samples were diluted to a final DNA concentration of 0,2 ng/µl before tagmentation of genomic DNA (gDNA). This step uses the Nextera transposome to tagment gDNA, which is a process that fragments DNA and then tags the DNA with adapter sequences in a single step. The next step amplifies the tagmented DNA by using a limited-cycle PCR program. The index primers and Nextera PCR Master Mix are added directly to the tagmented gDNA. After the amplification AMPure XP beads were used to purify the library DNA and to remove short library fragments.

Normalization of the libraries must be done to ensure more equal library representation in the pooled library. This can be done in in two different ways; Manually normalization or bead-based normalization. In current study, the former was used.

Pooling libraries combines equal volumes of normalized libraries in a single tube. After pooling, the library pool was diluted, denatured using NaOH, added 5 % PhiX and heat-denatured before loading the libraries for sequencing on a MiSeq platform (Illumina, USA) using a MiSeq v3 kit (Illumina, USA). The sample tagmentation, normalization and pooling of the libraries was performed by Lab Engineer Inga Leena Angell at MiDiv.

2.12 Bioinformatic analysis

2.12.1 Assembling, annotation and bacteriocin searching

The data obtained from the 16S rRNA gene sequencing was uploaded from GATC Biotechnology by using BioEdit Sequence Alignment Editor. The files with nucleotides were then copied and pasted into NCBIs BLAST tool to identify the bacterial strains.

The data obtained from the illumina MiSeq shotgun sequencing was assembled by Postdoc Jane Ludvigsen. Fasta files from the illumina MiSeq shotgun sequencing were uploaded in Geneious 8.1.7 where a "trim and filter" was done to remove quality lower than Q20 (99 % certainty of correct base). This was done to avoid unwanted overlap and bad quality of the genome. The genomes were assembled using SPAdes v3.9.0, with settings --careful and -k 127.

Further analyses of the data were done by using different bioinformatical tools and programs. Contigs in fasta format obtained from the assembling were uploaded in Rast (Rapid Annotation using Subsystem Technologies) to compare the different genomes. By selecting "Browse annotated genome in SEED viewer" a simple comparison of the genomes was done based on the "Organism Overview table".

For identification of possible bacteriocin genes in the genomes the bioinformatical tool Bagel4 was used. The DNA in fasta format were uploaded in Bagel4 to search for potential matches with structural bacteriocin genes. Gene topology of hypothetical bacteriocin gene clusters were made and illustrated by using CLC Workbench and performed by Senior Scientist Amar Telke.

2.12.2 Designing of primers and probes

Designing of primers and probes was performed in Geneious 8.1.7 and done by Postdoc Jane Ludvigsen. The designing was based on the identified structural gene of the bacteriocins obtained from bioinformatic analysis of the data from the illumina MiSeq Shotgun Sequencing of the bacterial genomes. The primers were ordered from Invitrogen by Thermo Fisher Scientific and the probes were ordered from Applied Biosystems® by life technologies, UK. Sequence specifications of the primers are described in table 2-1 and the probes are described in table 2-2.

3. Results

3.1 Selection of samples

The differences in how LGG colonizes the intestine of infants is of great importance in relation to the use of probiotics. The selection of samples was therefore based on an OUT screening of the ProPACT samples, with the aim to find OTUs with negative or positive correlation to LGG (Fig. 3-1).

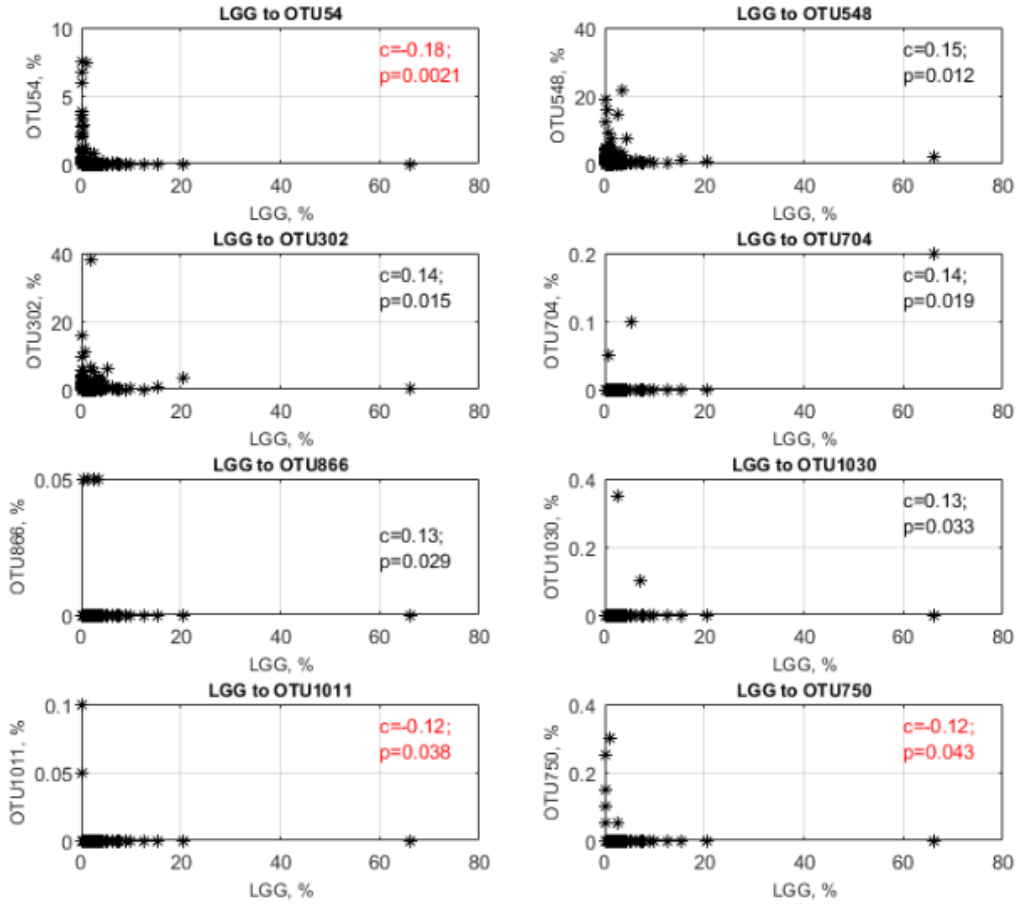

Figure 3-1 Correlation of LGG to different OTUs based on 16S rRNA gene sequence data obtained from the ProPACT cohort. From the correlation screening, 8 OTUs showed significant correlation to LGG. 5 OTUs with positive correlation and 3 OTUs with negative correlation. The correlation coefficient (c=) and p-value (p=) are marked in black for the positive correlations and red for the negative correlations.

As a result, the screening showed 8 OTUs, where 5 OTUs had significant positive correlation with LGG and 3 OTUs had significant negative correlation with LGG. With the criteria that mothers had received probiotics, stool samples from infants belonging to the 8 OTUs was identified and selected for further analysis. The subset of the stool samples from ProPACT used in this current study are listed in table 3-1.

Table 3-1 Description of the subset of stool samples from ProPACT used in this current study. The samples were selected based on the best negative or positive correlation with LGG.

Selected stool sample	Age category	OTU number	OTU %	LGG %	Correlation* (+/-)	Taxonomy (Genus)
1	10 days	704	0,2	66,15	+	*Streptococcus*
2	10 days	704	11,1	0,7	+	*Streptococcus*
3	10 days	104		20,5	+	*LGG*
4	90 days	548	15,6	0,35	+	*Streptococcus*
5	90 days	104		15,45	+	*LGG*
6	90 days	866	0,05	3,4	+	*Alloiococcus*
7	90 days	302	6,0	5,2	+	*Streptococcus*
8	1 year	1030	0,35	2,7	+	*Lactobacillus*
9	1 year	1030	0,1	7,1	+	*Lactobacillus*
10	2 years	750	0,15	0,05	-	*Abiotrophia*
11	2 years	750	0,3	1,0	-	*Abiotrophia*

*For correlation coefficient and p-value for the different OTUs see Fig. 3-1.

3.2 Optimization of culturing condition

Before screening for antimicrobial activity, an optimization of culturing condition of the stool samples was done. The desired amount of bacterial colonies to screen was between 100-1000 colonies per stool sample. After the first cultivation only 36 % (equal 4 of 11) of the samples showed growth on MRS agar. Therefore, BHI agar was tested as growth medium. This medium showed better growth, but several of the samples had gas production. The gas production resulted in bubbles in the agar which made it difficult to see possible inhibition zones. Therefore, the MRS media was used for the rest of the screening process with some adjustments in the dilution-series and incubation time.

3.3 Screening for bacteria with antimicrobial activity

Screening for bacteria with antimicrobial activity in the subset of stool samples from ProPACT was done to identify bacteria with potential inhibition mechanism against LGG. A multiple layer assay was used to detect antimicrobial activity in the form of visual inhibition zones. Fig. 3-2 illustrates the different steps in the screening for antimicrobial activity against the indicator LGG.

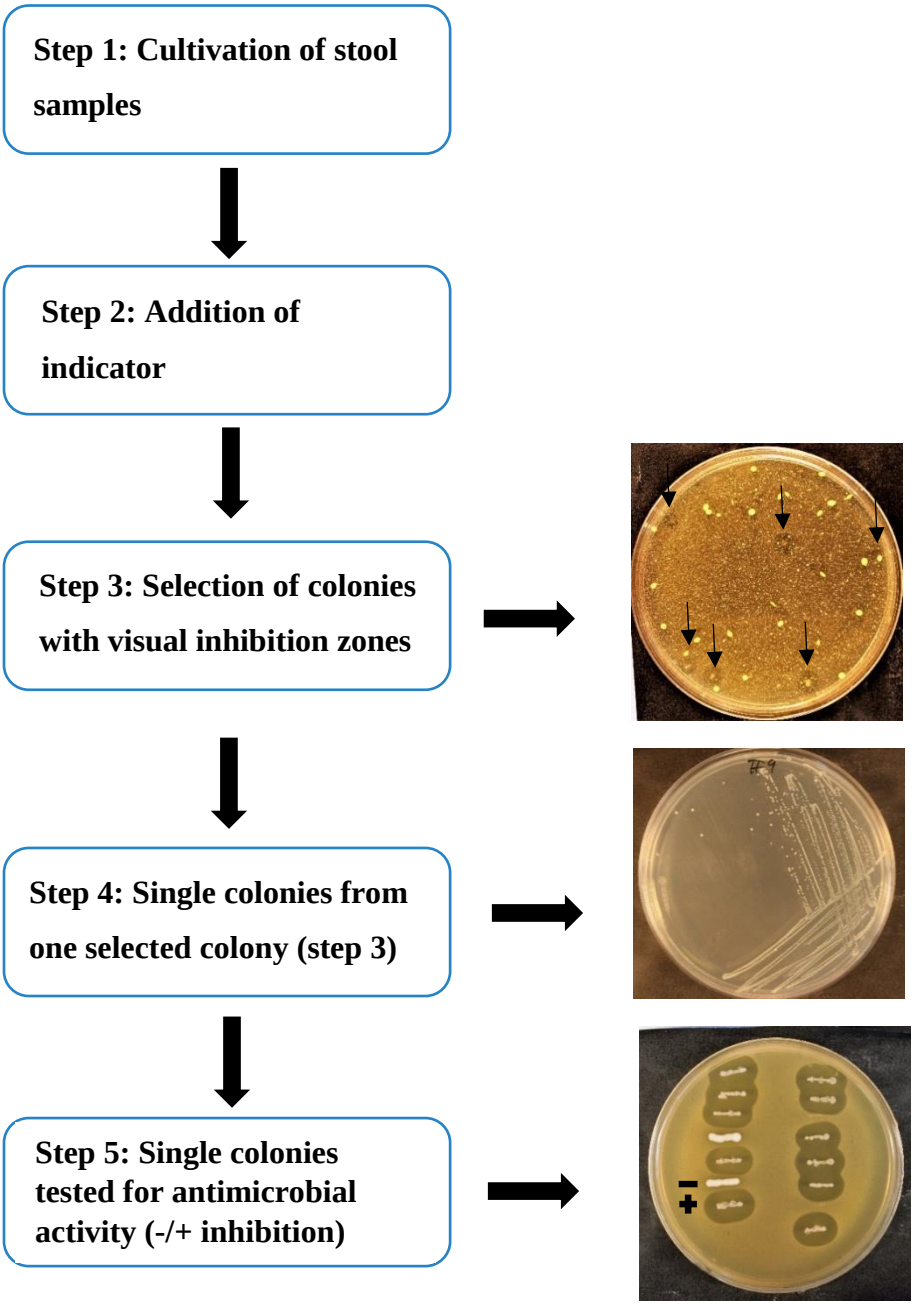

Figure 3-2 Screening for antimicrobial activity against indicator *Lactobacillus rhamnosus GG* with the use of a multiple layer assay.

After step 3 in the screening process, 27 % (equal 3 out of 11) of the stool samples showed visual inhibition zones against the indicator. Sample 6 and 7 had small and similar inhibition zones, while sample 8 had greater inhibition zones. Sample 6 had 12 % colonies with antimicrobial activity. Sample 7 had 7 % colonies with antimicrobial activity. Sample 8 had 1,2 % colonies with antimicrobial activity. The samples with antimicrobial activity are listed in table 3-2.

Table 3-2 Samples with visual antimicrobial activity against indicator *Lactobacillus rhamnosus GG*, and a description of single colonies selected for Sanger sequencing, test of inhibition spectrum and illumina MiSeq Shotgun Sequencing.

Stool sample	Total bacterial colonies with visual inhibition	Isolates selected for Sanger Sequencing[1]	Isolates used to test Inhibition Spectrum[2]	Isolates used for Shotgun Sequencing[3]
6	12 % (40 out of 312)	n = 3	n = 2	n = 1
7	7 % (9 out of 121)	n = 9	n = 2	n = 1
8	1.2 % (12 out of 1000)	n = 9	n = 2	n = 1

[1]Naming of the isolates selected for 16S rRNA gene Sanger Sequencing. Stool sample 6: P6-19 to P6-21. Stool sample 7: P7-10 to P7-18. Stool sample 8: P8-1 to P8-9.
[2]Naming of isolates used to test Inhibition Spectrum. Stool sample 6: P6-19 and P6-20. Stool sample 7: P7-17 and P7-18. Stool sample 8: P8-1 and P8-3.
[3]Naming of isolates used for illumina MiSeq Shotgun Sequencing. Sample 6: P6-20, sample 7: P7-17 and sample 8: P8-1.

3.3 Inhibition spectrum of antimicrobial activity

In order to determine if the antimicrobial activity had a narrow or a broad inhibition spectrum, a spot-on-lawn inhibition assay was done (see section 2.4 for details). Several of the single colonies with antimicrobial activity showed similar phenotypic character, in the manner of shape and color of bacterial colonies and sizes/types of visual inhibition zones. Therefore, only 2 isolates from each stool sample were selected to test the inhibition ability (table 3-2). The pure cultures from the same sample showed the same inhibition ability, while diverse inhibition pattern was found between the different stool samples. The two isolates from sample 8 showed an inhibition of approximately 80 % of the indicator strains that were tested. The isolates from sample 7 had an inhibition of approximately 42 %, while the isolates from stool sample 6 had an inhibition of approximately 44 % of the indicators. The isolates showed inhibition of different indicators such as *Pediococcus, Bacillus, Lactobacillus, Lactococcus, Staphylococcus, Listeria* and other *Enterococcus* strains. The results are summarized in table 3-3. For a detailed overview of all indicator strains tested and the results, see Appendix 2.

Table 3-3 Percentage inhibition of different genus in the spot-on-lawn inhibition assay to test the inhibition spectrum of the antimicrobial activity found in the stool samples.

Indicator (Genus)	Stool sample 6[1] Inhibition %*	Stool sample 7[2] Inhibition %*	Stool sample 8[3] Inhibition %*
Bacillus **	-	-	75 % (n=4)
Carnobacterium **	50 % (n=2)	-	100 % (n=2)
Enterococcus **	70 % (n=10)	60 % (n=10)	100 % (n=10)
Lacobacillus **	37.5 % (n=8)	50 % (n=8)	87.5 % (n=8)
Lactococcus **	100 % (n=3)	100 % (n=3)	100 % (n=3)
Leuconostoc **	100 % (n=1)	100 % (n=1)	100 % (n=1)
Listeria **	100 % (n=8)	25 % (n=8)	87,5 % (n=1)
Pediococcus **	33.33 % (n=3)	100 % (n=3)	100 % (n=3)
Staphylococcus **	-	-	40 % (n=10)
Streptococcus **	33.33 % (n=3)	-	33.33 % (n=3)
Total inhibition**	44 % (equal 25 of 56)	42 % (equal 24 of 56)	80 % (equal 45 of 56)

[1] Naming of isolates from stool sample 6: P6-19 and P6-20.
[2] Naming of isolates from stool sample 7: P7-17 and P7-18.
[3] Naming of isolates from stool sample 8: P8-1 and P8-3.
*No inhibition is marked in the table as " – "
***Total inhibition given as approximately percentage value based on all indicator strains tested.
**n is equal number of strains tested from the same genus.

Because BHI was selected as a medium through the spot-on-lawn assay, indicator LGG was tested once more on a BHI agar plate, and compared to the MRS agar plate. The isolates showed different inhibition pattern on the two agar plates. On the BHI agar plate the isolates from sample 7 and 8 showed similar inhibition zones, while the two isolates from sample 6 showed no activity against the indicator LGG. On the MRS agar plate, the two isolates from sample 8 were more effective and had greater zones than the isolates from sample 6 and 7.

3.4 Heat stability and Proteinase K sensitivity

Bacteriocins are small peptides and often heat stable, therefore biochemical analysis was performed to check the heat stability and proteinase K sensitivity of the antimicrobials. Based on the lack of visual inhibition zones on the agar plates, the antimicrobial activity was not detected after heat treatment. Only the unfiltered and non-heat treated samples showed antimicrobial activity. These samples were also Proteinase K sensitive when applied directly to the o/n-culture on the agar plate.

Nisin producer, *L.lactis* B1580 (K3) was included in all steps as a control because of its heat stability and Protinase K sensitivity. K3 showed antimicrobial activity after all treatments (see section 2.5 for detailes) .

3.5 Quantification of antimicrobials in liquid

To see if the antimicrobial substance was produced in liquid, a microtiter assay was performed. All the samples had aliquots with different treatment, but none of them showed visible or detectible inhibition of LGG in liquid when using BHI as a growth medium on the microtiter plate. At the MRS plate only the concentrated sample inhibited the growth of LGG. The visible inhibition and the absorbance values showed 4 wells with antimicrobial activity. One unit of bacteriocin activity (BU) is defined as the amount of bacteriocin required to produce 50 % growth inhibition (Holo et al. 1991). An inhibition of the indicator LGG in 4 wells means that the BU/ml in the original supernatant is equal 80 BU/ml.

3.6 Sequencing for identification of potential Inhibition mechanism

3.6.1 16S rRNA gene Sanger Sequencing

The isolates selected from the antimicrobial screening (table 3-1), were sent to GATC Biotech for 16S rRNA gene sequencing to identify the producer species. After sequencing 20 of 21 samples showed a query coverage between 84-100 % nucleotides and between 96-99 % identity to the bacteria species *Enterococcus faecalis*. A full overview of the isolates and identity score can be found in Appendix 3.

3.6.2 Illumina MiSeq Shotgun Sequencing

With the aim to identify potential inhibition mechanism in the bacterial genomes, an illumina MiSeq Shotgun Sequencing were performed. With a coverage of minimum 50x for each genome together with the number of contigs obtained after assembly, N50 and L50 values (Table 3-4) it can be stated that a deep sequencing with high quality were obtained. Rast and The Seed Viewer was used to compare the three genomes and the comparison showed differences in subsystems such as cell wall components, DNA-metabolism, transporter and repair systems, fermentation, resistance among more. A general organism overview of the three bacterial genomes are described in table 3-4.

Table 3-4 Organism overview for the three genomes sequenced by illumina MiSeq Shotgun Sequencing.

Samples	**P6-20**[1]	**P7-17**[2]	**P8-1**[3]
Domain	Bacteria	Bacteria	Bacteria
Genome	*Enterococcus*	*Enterococcus*	*Enterococcus*
Taxonomy	*E.faecalis*	*E.faecalis*	*E.faecalis*
Closest neighbors*	*E.faecalis TX0104 (score 501)*	*E.faecalis V583 (score 521)*	*E.faecalis V583 (score 538)*
Size	2 822 964	2 916 256	3 183 391
GC content	37.5	37.4	37.0
Number of Contigs	88	80	58
N50	108 299	329 229	514 562
L50	10	4	3
Number of subsystems	360	360	364
Number of coding sequences	2654	2786	3038

*Based on The Seed Viewer version 2.0 highest score of closest neighbors.
[1] Sequenced isolate belonging to stool sample 6.
[2] Sequenced isolate belonging to stool sample 7.
[3] Sequenced isolate belonging to stool sample 8.

3.6.3 Identification of bacteriocin genes

A potential inhibition mechanism that can explain the differences in colonization of LGG in infants, could be bacteriocin production. A search for bacteriocin genes in the genomes was done with the bioinformatical tool Bagel4. This resulted in identification of bacteriocin Enterolysin A in P6-20 and P7-17, and the bacteriocin Enterocin EJ97 was identified in P8-1. These two bacteriocins had a 100 % match in the structural gene sequence. Several matches of other bacteriocins were also found in P8-1. The two-component lytic system Cytolysin, showed a 98 % match. “Bacteriocin A” shared 62 % protein identity with Enterocin NKR-5-3B, and “Bacteriocin B” shared 82 % protein identity with Enterocin SE-K4. The gene topology of the different bacteriocin gene clusters is shown in Fig. 3-6. The sequences used for generation of the bacteriocin gene clusters can be found in Appendix 4.

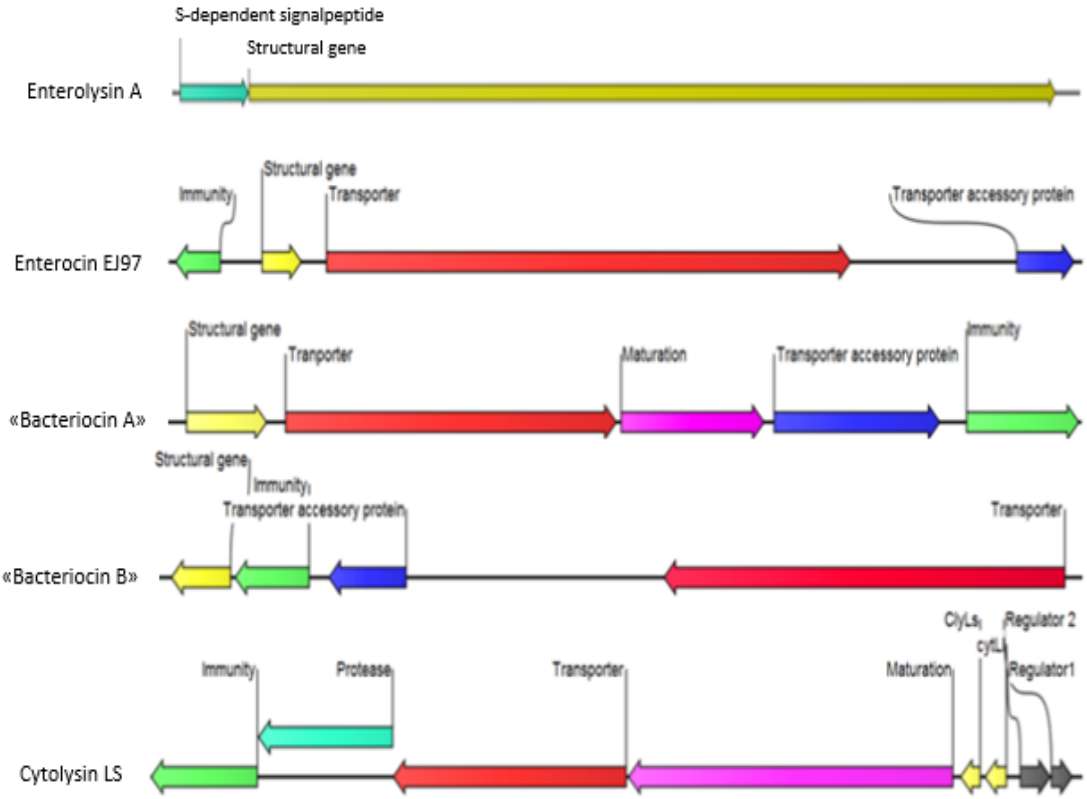

Figure 3-3 Bacteriocin gene clusters found in the three *Enterococcus faecalis* genomes. Structural gene in yellow, immunity gene in green, transporter gene in red, maturation gene in purple, accessory protein in blue and signal peptide in aqua.

3.7 Correlation Analysis

3.7.1 Preparation for qPCR screening

Searching for bacteriocin genes in the bacterial genomes resulted in several matches. For the qPCR screening Enterolysin A and Enterocin EJ97 were selected. Before the qPCR screening a gradient PCR was performed to find the optimal annealing temperature for the EntA and EJ97 specific primers. There was no difference in the band quality on the agarose gel due to the different temperatures (49-64 ^{o}C) after the gradient PCR. All the isolates from stool sample 6, 7 and 8 (table 3-1) were run on a regular PCR for detection of the bacteriocin genes. EntA was detected in all the isolates from sample 6 and 7, and all the isolates from sample 8 showed detection of EJ97.

3.7.2 qPCR screening

A subset of stool samples from ProPACT included their respective mother-child pair (Appendix 1) was used in the qPCR screening. In total 60 samples with extracted DNA from

ProPACT was used in the screening for EJ97, EntA, LGG, 16S rRNA and *Enterococcus*. This quantitative analysis was done in order to look for positive or negative correlations between the bacteriocin producing bacteria and LGG.

Only 10 % (6 of 60) of the DNA samples showed detection of either EJ97 or EntA (see Appendix 1 for details). The correlation analysis was therefore based on the relative amount of LGG and Enterococcus found in the DNA samples. Pearson- and Spearman correlation coefficient was calculated to respectively 0,559 and 0,439. This indicates approximately 50 % positive correlation between LGG and Enterococcus (Fig. 3-4).

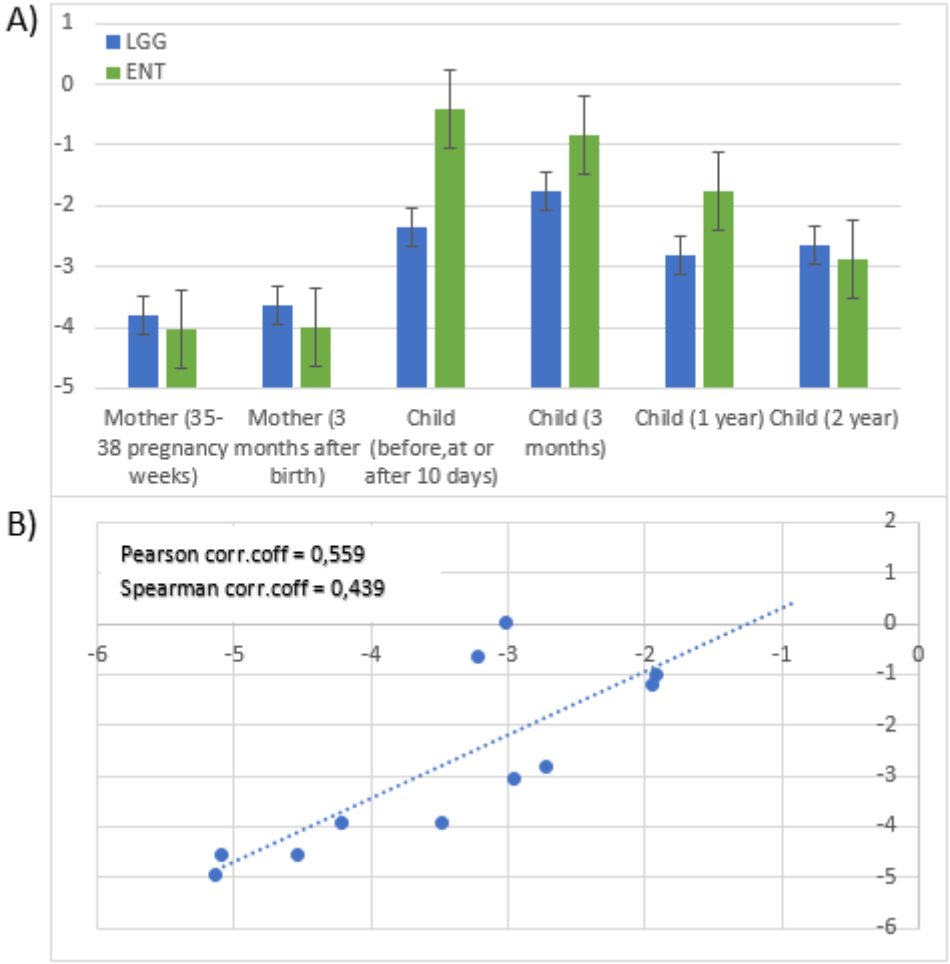

Figure 3-4 A) Presence of LGG and Enterococcus in the DNA samples from the mother and children-pair (ProPACT). The data is based on the relative amount of bacteria present after the qPCR screening. B) Illustrates the correlation between Enterococcus and LGG in a logarithmic scale. The data is based on the relative amount of bacteria present after the qPCR screening.

In addition to the qPCR screening, a known Enterolysin A producer and a synthetic Enterocin EJ97 were tested to compare the visual inhibition zones with the visual inhibition zones obtained from the stool sample isolates (table 3-1). This was done to get an indication that the inhibition of LGG was caused by these specific bacteriocins. The known Enterolysin A

producer and the two samples P7-17 and P6-20 with potential Enterolysin A activity showed similar inhibition zones. On the other hand, the synthetic Enterocin EJ97 did not give any visual inhibition zone against the indicator LGG but worked perfectly on the control plate with indicator *Enterococcus faecium*.

4. Discussion

The main finding in this thesis was the detection of bacteria with antimicrobial activity from infants gut that could inhibit the growth of LGG. This is a proof of principle that contributes to recent evidence where it is suggested that the high variability in the effects of probiotics on the host or its microbiome is due to person-specific mucosal colonization resistance (Zmora et al. 2018). Furthermore, several putative bacteriocins were found in the bacterial genomes. Production of bacteriocins by LAB has generally been considered as a probiotic trait, but few studies have assessed their probiotic effect and impact on the normal gut microbiota (Umu et al. 2016).

4.1 Identification of bacteria with antimicrobial activity

After cultivation and isolation of the bacteria with antimicrobial activity, the 16S rRNA gene Sanger Sequencing revealed that all the isolates were belonging to the species *E. faecalis* (Appendix 3). *Enterococci* can be found in a variety of environments such as water, soil, food, animals and humans. They are also among the most dominant LAB in the intestinal flora (Nes et al.,2007) which may explain the high prevalence in the stool samples.

However, none of the other bacteria genera found in the first correlation screening (Table 3-1), was detected after cultivation and 16S rRNA gene Sanger Sequencing. This can be explained by the culturing condition. *Streptococcus, Alliococcus* and *Abiotrophia* was some of the genera related to the OTUs with an association to LGG. While *Alloiococcus* strains require aerobic atmospheric growth conditions (Miller et al. 1996), most *Streptococci* and *Abiotrophia* are facultative anaerobes. Some *Streptococci* are strict anerobes and most stains from these genera require enriched growth media such as blood agar to be cultivated (Sherwood et al. 2014). Furthermore, *E. faecalis* have the ability to metabolically adapt to an oligotrophic environment (Hartke et al. 1998). This might explain that *E. faecalis* is more suitable for cultivation compared to other bacteria.

The illumina MiSeq Shotgun Sequencing and the bioinformatical analysis (Table 3-4) corresponds to recent findings of comparative genomic analysis of *E. faecalis* where the genomes had a GC content ranging from 37.0 % to 38.0 % and an average genome size of 2.94 ± 0.15 Mb (Wang et al. 2018). Furthermore, the differences between the genomes such as genome size, number of contigs, GC content, coding sequences and subsystems, together with the different inhibition ability obtained from the inhibition spectrum assay (Appendix 3), may indicate that the antimicrobial activity are produced by three different *Enterococcus*

strains. To distinguish between the strains, it is possible to do a phylogenetic analysis by using other bioinformatical tools such as MUSCEEL and Gblocks (Wang et al. 2018). It is also possible to use a Pulse Field Gel Electrophoresis (PFGE). This type of electrophoresis consists on periodically changing of the orientation of the electric field. This enabling separation of high-molecular-weight fragments and with the use of PFGE rare-cutting restriction endonucleases, a low number of fragments generates, resulting in a banding pattern that is easy to interpret. PFGE is a highly discriminatory and reproducible method and has been used to differentiate strains of *E. faecalis* and important probiotic bacteria (Holzapfel et al.,2001).

4.2 Identification of potential inhibition mechanism

Many bacteriocins from *Enterococci* have been purified and genetically characterized, most of them obtained from *E. faecium* and *E. faecalis*. These bacteriocins belongs almost exclusively to the heat-stable, non-lantibiotic class II. The exception is the two-peptide lantibiotic cytolysin found in *E. faecalis* (Nes et al.,2007).

To test the purity and the activity of the isolates, a spot-on-lawn inhibition assay were performed. This assay was also used to test the inhibition ability with the use of several indicators and the isolates showed different inhibition ability (Appendix 2). The isolates showed an inhibition of between 42-80 % of all indicator strains tested (Table 3-3). This can might be explained by the action of a broad-spectrum bacteriocin.

The illumina MiSeq Sequencing of the bacterial genomes and bioinformatical analysis, showed that several bacteriocin genes were present in the genomes. The bacteriocin Enterolysin A was found in sample P6-21 and P7-17 and had a 100 % match in the structural gene. The isolates from these genomes showed similar inhibition zones when tested on MRS agar with LGG as indicator. In addition, these isolates also showed similar inhibition zones with the control strain with Enterolysin A activity (*E. faecalis*, LMG2333). This may indicate that the antimicrobial activity from sample P6-21 and P7-17 is due to the bacteriocin Enterolysin A.

Furthermore, bioinformatical analysis of the genome P8-1 revealed that several bacteriocin genes were present (Fig. 3-3). This may explain that the isolates from this sample showed the biggest inhibition zones against indicator LGG and had the broadest inhibition spectrum, approximately 80 %, when tested against different indicator strains (Table 3-3). A 100 % match in the structural gene of Enterocin EJ97 were found in the P8-1 genome. The

bacteriocin Enterocin EJ97 is a peptide produced by *E. faecalis* and is active against several G+ bacteria, including enterococci and species of *Listeria, Bacillus* and *Staphylococcus* (Sánchez-Hidalgo et al. 2003). However, the synthetic Enterocin EJ97 was tested and did not give any visual inhibition zone against the indicator LGG but showed antimicrobial activity on the control plate with indicator *Enterococcus faecium*. This indicates that the inhibition of LGG could be caused by a different antimicrobial substance than Enterocin EJ97.

The enterococcal cytolysin were also detected in genome P8-1 with a 98 % match in the structural gene. This is a two-peptide lytic system relative to a large family of toxins and bacteriocins secreted by both pathogenic and non-pathogenic G+ bacteria included *E. faecalis* (Coburn et al. 2003). Furthermore, the last two bacteriocins found in P8-1 showed a lower degree of identity match in the structural gene. “Bacteriocin A” shared 62 % protein identity with Enterocin NKR-5-3B, and “Bacteriocin B” shared 82 % protein identity with Enterocin SE-K4. Enterocin NKR-5-3B is classified as a circular bacteriocin produced by *E. faecalis* and *E. facium* and inhibits strains of *Bacillus, Listeria, Pediococcus, Lactobacillus* and *Lactococcus* (Bactibase, 2017). Enterocin SE-K4 is also produced by *E. faecalis* and belonging to the class IIa bacteriocins and is active against strains of *Enterococcus, Bacillus, Clostridium* and *Listeria* (Eguchi et al. 2001). However, the low identity match may indicate that the “Bacteriocin A” and “Bacteriocin B” differs from the already known Enterocin NKR-5-3B and Enterocin SE-K4.

Hypothetical gene clusters were made from the sequences obtained from the illumina MiSeq Shotgun Sequencing (Appendix 4) and included the structural gene, immunity gene, transporter gene, maturation gene and accessory gene (Fig. 3-3). These genes, together with other regulatory genes, are usually in operon clusters and are involved in the production of active bacteriocins (Cleveland et al. 2001).

Today, antibiotic resistance is one of the biggest threats to global health, food security and development (WHO, February 2018). Consequently, there is an urgent need for new antimicrobial agents. In general, bacteriocins have no or low toxicity toward eukaryotic cells and can be beneficial for the gut microbiota (Umu et al.,2016). However, the cytolysin operon is encoded on mobile elements and is lethal to a broad range of prokaryotic and eukaryotic cells (Coburn et al. 2003). Even though many bacteriocins have been discovered, more research is needed to fully understand the potential of bacteriocins in food, feed and regarding human health.

4.3 Correlation analysis

Correlation is a statistical technique that can show whether and how strongly pairs of variables are related. In this study the correlation analysis revealed a positive correlation of approximately 50 % between *Enterococcus* and LGG (Fig. 3-4). The qPCR screening was done on a selected subset of stool samples from ProPACT including their respective mother-child pair (Appendix 1). In total 60 samples with extracted DNA from ProPACT was used in the screening for Enterocin EJ97, Enterolysin A, LGG, 16S rRNA and *Enterococcus*. The bacteriocins Enterolysin A and Enterocin EJ97 were only detected in a few samples (approximately 10 %). Therefore, *Enterococcus*, that after sequencing was detected as a bacterial producer of the potential antimicrobial activity, was included in the screening.

First, it was assumed that the antimicrobial inhibition of LGG could might be reflected as a negative correlation with LGG. However, the detection of a positive correlation may indicate a form of dependency between these two variables. The gut microbiota is a complex niche with numerous of different bacteria competing for common resources. Therefore, the bacteria must develop strategies to persist in the gut. Besides the ability to inhibit competitors it is also important for the bacteria to coexist with other gut inhabitants and the host in the dynamic nature of a healthy gut. This may explain that the dominant *Enterococcus* found in the samples in the current study had a positive correlation to LGG. Both *Enterococcus* and LGG are LAB with the ability to persist in the GI tract. And most likely they are selected in the same way under the same conditions, dependent on factors such as available nutrition, before competition is necessary. Furthermore, the first OTU screening, showed a majority of OTUs with a positive correlation with LGG (Table 3-1). The samples selected for further analysis in current study belonged to approximately 80 % of these positive correlated OTUs, and can therefore be a part of the explanation of the positive correlation found between LGG and *Enterococcus*.

Unfortunately, little is known about this kind of positive correlation between LGG and *Enterococcus*. Most studies have shown that there is a negative association between LGG and *Enterococcus* strains such as *E.* faecium. Vancomycin-resistant enterococci (VRE) have become a major threat and *E.* faecium is of special concern since it can easily acquire new resistances and is an excellent colonizer of the human GI tract (Tytgat et al. 2016). And it is believed that LGG supplementation temporarily eliminates the VRE carrier state and increases GI counts of *Lactobacillus* (Szachta et al. 2011). However, another study about the relationship between *L. rhamnosus* and *E. faecalis* during biofilm formation showed that *L. rhamnosus*

enhanced the growth of *E. faecalis in vitro* (Montecinos et al. 2016). This positive association in the biofilms differs from other studies. However, different experimental conditions such as *in vivo* versus *in vitro* assessment must be taken in consideration.

Previously it has been suggested that bacteriocins play important roles to allow different bacteria to establish a long-term commensal relationship with human hosts (Zheng et al. 2014). However, only few studies provide evidence for an ecological relevance for bacteriocin production by human commensal microbiota. Bacteriocins may help the producer to invade new niche by competitive exclusion of other inhabitants, usually closely related bacteria, but in general it seems like the main structure of the composition of bacteria in the gut is relatively resilient to the administration of LAB in mice (Umu et al. 2016). In the current study it was detected a low prevalence of bacterial colonies with antimicrobial activity against LGG (Table 3-2). However, under certain conditions this antimicrobial activity may influence the establishment of different bacteria such as LGG in the microbiota. It must also be mention that the *in vitro* activity of bacteriocins may differ from the once *in vivo*. This can be explained by innate gene regulation in a more complex environment (Umu et al. 2016).

4.4 Technical considerations

As a part of the cultivation optimization, BHI medium was tested as a growth medium. This resulted in several samples with gas production on the agar plates which made it difficult to see potential inhibition zones. The BHI medium support growth of a wide range of microorganisms. A possible explanation of the gas production could be to the growth of coliforms. Coliforms are members of the family *Enterobacteriaceae* and include a wide range of bacteria. They are defined as facultative anaerobic that ferment lactose with gas formation, and make up 10 % of the intestinal microorganisms of humans and other animals (Sherwood et al.,2014).

The differences in how the isolates inhibited LGG dependent on media (MRS vs BHI) can be explained by inhibiting or inducing components in the medium that can affect the antimicrobial production. One possible way of inhibition can be due to components in the medium that interferes with the quorum sensing system and affects the bacteriocin production (Renye et al.,2016). Furthermore, analysis of heat stability and Proteinase K sensitivity was performed to test the character of the antimicrobials. The filtration and heat treatment resulted in no visible inhibition zones, while the isolates were sensitive of Proteinase K. The sensitivity of Proteinase K indicates that the antimicrobial activity is caused by something

proteinaceous, and by ammonium sulfate precipitation the potential proteinaceous antimicrobial can be concentrated. The fact that the concentrated sample showed activity only in liquid when tested in a microtiter plate and not at the agar plate, could be explained by the difference in concentration/sample volume (100 µl in liquid and 10 µl applied on agar plate). While a potential explanation of the loss of antimicrobial activity after filtration might be the hydrophobicity of the bacteriocin which make it adhere to the surface of the producer. The fact that the antimicrobial activity from the isolates were not detected in liquid on agar plates corresponds to another study of Enterolysin A where the bacteriocin production initially was observed only on solid media and not in culture supernatants (Nilsen et al. 2003). Bacteriocin production is growth associated and often strictly regulated by the quorum sensing system. However, the yield of bacteriocin produced is affected by several factors such as growth media and fermentation condition (Parente et al.,1999). Inhibition spectrum, biochemical analysis and quantification of the antimicrobial activity was just a side part in this master thesis and should be repeated considering factors such as fermentation condition (dilution rate, pH, temperature), selection of growth medium and incubation time.

Furthermore, only a subset of stool samples from the ProPACT cohort were used in this thesis, both in the screening for antimicrobial activity and the qPCR screening. If all the samples could be included, it is believed that the results would give more strength to the correlation analysis as well as a better picture of the prevalence of bacterial colonies with antimicrobial activity.

4.4 Concluding remarks and future perspectives

LGG is believed to have an important role in preventing allergic inflammatory diseases and to restore and maintain the natural balance of good bacteria in the gut. In this thesis it was succeeded to isolate bacteria with antimicrobial activity against LGG from the subset of stool samples from the ProPACT cohort. The bacteria with antimicrobial activity was identified as *E. faecalis* and antimicrobial activity may be due to the production of several putative bacteriocins found in the bacterial genomes. However, which bacteriocin and if it's truly active against LGG is still unknown. A suggestion for further work is to purify concentrated liquid culture from the isolates with antimicrobial activity found in the current study. The purification can be done by cation exchange chromatography and hydrophobic interaction chromatography followed by mass spectrometry to identify the bacteriocin (Guyonnet et al.,2000). With a separation and identification of the bacteriocin, the active fraction can be tested again with a spot-on-lawn inhibition assay to see if it's still active or if the inhibition is

caused by another antimicrobial agent. With identification of the potential inhibiting bacteriocin or the bacterial strain, a new and more specific correlation analysis could be performed. It is also possible to perform a knockout study of the different bacteriocin genes and observe the phenotypic character as visible inhibition zone against LGG.

Furthermore, a positive correlation was found between *Enterococcus* and LGG. This may indicate that in the complex niche of microbiota these bacteria coexist and somehow is dependent on each other. However, the qPCR screening of LGG and *Enterococcus* done in this study represented only a subset of the total stool samples from the ProPACT cohort. Also, it must be taken in consideration that the qPCR screening represented the most dominant *Enterococcus* present. If a more specific screening for *E. faecalis* could be performed, the results might be different. Another potential way to further investigate the positive correlation is with the use of mice models. With feeding of both LGG and *E. faecalis* over a certain period of time, a new analysis of the microbial composition and a correlation analysis between these could be performed. It is also interesting to look for differences in colonization under treatment with antibiotic which may cause alteration of the microbiota. This type of alteration of the microbiota can create a new niche in the intestine, where the low prevalence of competitive enterococcal bacteriocins might can dominate more.

As a final conclusion, the results presented in this thesis improved our understanding of potential inhibition mechanisms against the probiotic strain LGG and supports the hypothesis that the effect of probiotics can be dependent on the intrinsic microbiota. However, the results deserves future investigation, as a deeper insight into the inhibition mechanisms can serve a key role and be in great importance in relation to the future use of probiotics.

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Appendix

Appendix 1: Overview of samples from ProPACT used in qPCR screening for EntA, EJ97, 16S rRNA, LGG and Enterococcus

ProPACT number	Stool samples from mother/child	ProPACT number	Stool samples from mother/child
212-22205	Mother 35-38 pregnancy weeks	212-21853	Mother 35-38 pregnancy weeks
213-22205	Mother 3 months after birth	213-21853	Mother 3 months after birth
223-22205*	Child before, at or after 10 days	223-21853*	Child before, at or after 10 days
225-22205	Child at 3 months (90 days)	225-21853*[2]	Child at 3 months (90 days)
226-22205	Child at 1 year	226-21853	Child at 1 year
212-21116	Mother 35-38 pregnancy weeks	227-21853	Child at 2 years
213-21116[6]	Mother 3 months after birth	212-22341	Mother 35-38 pregnancy weeks
223-21116*	Child before, at or after 10 days	213-22341	Mother 3 months after birth
225-21116	Child at 3 months (90 days)	223-22341	Child before, at or after 10 days
226-21116[3]	Child at 1 year	225-22341*[4]	Child at 3 months (90 days)
227-21116	Child at 2 years	226-22341	Child at 1 year
212-23108	Mother 35-38 pregnancy weeks	227-22341	Child at 2 years
213-23108	Mother 3 months after birth	213-23431	Mother 3 months after birth
223-23108	Child before, at or after 10 days	223-23431	Child before, at or after 10 days
225-23108*	Child at 3 months (90 days)	225-23431	Child at 3 months (90 days)
226-23108	Child at 1 year	226-23431*	Child at 1 year
227-23108	Child at 2 years	227-23431	Child at 2 years
212-22764	Mother 35-38 pregnancy weeks	212-22004	Mother 35-38 pregnancy weeks
213-22764	Mother 3 months after birth	213-22004	Mother 3 months after birth
223-22764	Child before, at or after 10 days	223-22004	Child before, at or after 10 days
225-22764*	Child at 3 months (90 days)	225-22004	Child at 3 months (90 days)
226-22764	Child at 1 year	226-22004[1]	Child at 1 year
227-22764	Child at 2 years	227-22004*	Child at 2 years
212-22215	Mother 35-38 pregnancy weeks	212-22821	Mother 35-38 pregnancy weeks
213-22215	Mother 3 months after birth	213-22821	Mother 3 months after birth
223-22215	Child before, at or after 10 days	223-22821	Child before, at or after 10 days
225-22215[5]	Child at 3 months (90 days)	225-22821	Child at 3 months (90 days)
226-22215*	Child at 1 year	226-22821	Child at 1 year
227-22215	Child at 2 years	227-22821*	Child at 2 years

*Samples used in the antimicrobial screening (for more information see table 3-1). [1]Detection of EJ97 with cq value 35,00. [2]Detection of EntA with cq value 29,94. [3]Detection of EntA with cq value 35,00. [4]Detection of EntA with cq value 35,00. [5]Detection of EntA with cq value 35,00. [6]Detection of EntA with cq value 35,00.

Appendix 2: Inhibition spectrum

A1 and A3 is from sample 8, A17 and A18 is from sample 7 and A19 and A20 is from sample 6. The controls included is K1: Enterococcus faecium, Enterocin A og B (T136), K2: Enterococcus faecium L50.1 Bacteriocin L.cintas and K3: LMG2122 Nisin, B1580. The capital G means transparrent inhibition and D means diffuse inhibition.

	Stool Sample 8		Stool Sample 7		Stool Sample 6			Controls	
Indikator	P8-1	P8-3	P7-17	P7-18	P6-19	P6-20	K1	K2	K3
LGG (MRS)	2	2	1	1	1	1	0	0	3
LGG (BHI)	2	2	2D	2D	0	0	0	1	3
*Bacillus cereus (LMGT2805)**	0.5G	0.5G	0	0	0	0	0.5G	0.5G	0.5G
Bacillus cereus ATCC 9139 B	1G	1G	0	0	0	0	0	0.5G	1G
Bacillus cereus 1230, Granum11-91	0.5G	0.5G	0	0	0	0	0	0.5G	0.5G
B.cereus ATCC 2 (Matforsk)	0	0	0	0	0	0	0	0	1
Carnobacterium divergens NCDO 2306	0.5	0.5	0	0	0	0	2	2	1
Carnobacterium pisciola	0.5G	0.5G	0	0	0.5G	0.5G	0	0.5G	0.5G
Enterococcus avium	1	1	1D	1D	1	1	2	0.5	1
*Enterococcus faecalis 2333**	0.5	0.5	0	0	0.5	0.5	0.5D	1	0.5G
Enterococcus faecalis 3088	1	1	0.5	0.5	1	1	2	2	0.5
Enterococcus faecalis 158B	1	1	0	0	0	0	0.5	1	0.5
Enterococcus faecalis 111a	1	1	0	0	0	0	0.5	1	0
Enterococcus faecalis 29C	0.5	0.5	0	0	0	0	0	0	0
*Enterococcus faecium 2763**	1	1	0.5	0.5	0.5	0.5	2	0	1
Enterococcus faecium 2772	1	1	1	1	1	1	2	0.5	1
Enterococcus faecium 2783	1	1	1D	1D	1	1	0	2	1
Enterococcus faecium 2876	1	1	1	1	1	1	2	0	1
Lactobacillus curvatus 2353	2	2	1D	1D	0.5D	0.5D	2	2	3
Lactobacillus curvatus 2355	2D	2D	0	0	0	0	1D	1	3D
Lactobacillus plantarum 2003	1	1	2D	2D	1	1	0	1	1
Lactobacillus plantarum 2352	2	2	0	0	0	0	1	1	3
Lactobacillus plantarum 3125	1	1	0	0	0	0	0	0	2
Lactobacillus sakei 2361	2	2	2	2	1	1	2	2	3
Lactobacillus sakei 2380	1	1	1	1	0	0	2	2	3
Lactobacillus salivarius	0	0	0	0	0	0	0.5G	0	0.5G
Lactococcus garvieae	1	1	0.5	0.5	1	1	2	1	2
Lactococcus lactis IL1403	2	2	2D	2D	2	2	0	1	2
Lactococcus lactis 2081	1	1	1	1	1	1	0	1	0.5
Leuconostoc gelidium	1	1	1D	1D	1	1	0.5D	1	0.5
Listeria innocua 2710	0.5D	0.5D	0	0	0.5D	0.5D	2D	2D	0.5
Listreia innocua 2785	1	1	0.5D	0.5D	0.5	0.5	0	2	1
Listeria ivanovii	2	2	0.5	0.5	0.5	0.5	3	3	2
Listeria monocytogenes 2604	1	1	0	0	0.5	0.5	0	1	0.5
Listeria monocytogenes 2650	0	0	0	0	0	0	0	0	0
Listeria monocytogenes 2651	1	1	0	0	0	0	2	2	0.5
Listeria monocytogenes 2652	1	1	0	0	0	0	2	2	1D
Listeria monocytogenes 2653	1	1	0	0	0	0	2	2	0
41 Pediococcus acidilactici	1	1	3D	3D	1	1	2	0	0
Pediococcus pentosaceus 2001	1	1	3	3	0	0	3	0.5	0.5
Pediococcus pentosaceus 2366	1	1	2D	2D	0	0	1D	0.5	2
Staphylococcus aureus 3022	0	0	0	0	0	0	0	0	1
Staphylococcus aureus 3023	0	0	0	0	0	0	0.5	0	1
Staphylococcus aureus 3242	0	0	0	0	0	0	0	0	1
Staphylococcus aureus 3262	0.5	0.5	0	0	0	0	0	0	1
Staphylococcus aureus 3263	0	0	0	0	0	0	0.5	0	1
Staphylococcus aureus 3264	0	0	0	0	0	0	0	0	1
Staphylococcus aureus 3265	0.5	0.5	0	0	0	0	0	0	1
staphylococcus aureus 2723	0.5	0.5	0	0	0	0	2	0	0
staphylococcus aureus 3255	0.5G	0.5G	0	0	0	0	0.5G	0	1G
staphylococcus aureus 4015	0	0	0	0	0	0	1	0	0
Strep agalactiae Val	0	0	0	0	0	0	0	0	0
Str.dysgalactiae 3890	0	0	0	0	0	0	0	0	0
Str.dysgalactiae 3899	0.5	0.5	0	0	0.5D	0.5D	1	1	2
K1	1	1	1	1	1	1	0	2	0.5
K2	1	1	1D	1D	0.5	2	0	1	
K3	2	2	2D	2D	2	2	0	1	0

Appendix 3: 16S rRNA sequencing results

Stool sample	Isolate	Species	Query Cover (%)	Identity (%)
8	P8-1	*Enterococcus faecalis*	100	97
8	P8-2	*Enterococcus faecalis*	97	98
8	P8-3	*Enterococcus faecalis*	99	98
8	P8-4	*Enterococcus faecalis*	91	98
8	P8-5	*Enterococcus faecalis*	99	99
8	P8-6	*Enterococcus faecalis*	85	98
8	P8-7	*Enterococcus faecalis*	98	99
8	P8-8*	*Enterococcus faecalis*	-	-
8	P8-9	*Enterococcus faecalis*	87	99
7	P7-10	*Enterococcus faecalis*	99	98
7	P7-11	*Enterococcus faecalis*	88	98
7	P7-12	*Enterococcus faecalis*	75	96
7	P7-13	*Enterococcus faecalis*	85	97
7	P7-14	*Enterococcus faecalis*	96	98
7	P7-15	*Enterococcus faecalis*	96	96
7	P7-16	*Enterococcus faecalis*	84	98
7	P7-17	*Enterococcus faecalis*	87	98
7	P7-18	*Enterococcus faecalis*	99	99
6	P6-19	*Enterococcus faecalis*	98	98
6	P6-20	*Enterococcus faecalis*	99	98
6	P6-21	*Enterococcus faecalis*	100	99

*No Sequencing result.

Appendix 4: DNA Sequences used for generation of putative bacteriocin gene clusters

>Enterolysin A

AGGAGGGGTTTCTATGAAAAATATTTTACTTTCTATTCTAGGGGTATTATCTATCGTTGTTTCTTTGGCGTTTTCTTCTTATTCT
GTCAACGCAGCTTCTAATGAGTGGTCGTGGCCACTGGGCAAACCATATGCGGGAAGATATGAAGAAGGACAACAATTCGGGA
ACACTGCATTTAACCGAGGAGGTACTTATTTCCATGATGGGTTTGACTTTGGTTCTGCTATTTATGGAAATGGCAGTGTGTATG
CTGTGCATGATGGTAAAATTTTATATGCTGGTTGGGATCCTGTAGGTGGAGGCTCATTAGGTGCATTTATTGTACTACAAGCG
GGAAACACAAATGTGATTTATCAAGAATTTAGCCGAAATGTTGGAGATATTAAAGTTAGCACTGGACAAACTGTTAAAAAAG
GACAGCTGATAGGAAAGTTTACTTCTAGTCATTTACATTTAGGAATGACAAAAAAAGAATGGCGTTCTGCTCATTCTTCTTGG
AATAAAGATGATGGCACTTGGTTTAACCCAATTCCTATACTTCAAGGAGGATCTACGCCTACGCCTCCAAATCCAGGACCAAA
AAATTTCACAACAAATGTTCGTTACGGATTGCGGGTCCTCGGAGGTTCATGGTTACCAGAAGTAACCAACTTTAACAATACCA
ATGATGGTTTCGCAGGTTACCCTAATCGTCAACATGATATGCTTTATATAAAGGTAGATAAAGGGCAAATGAAATATCGTGTT
CACACGGCTCAAAGCGGATGGTTGCCTTGGGTAAGTAAAGGGGATAAGAGCGATACAGTAAATGGAGCGGCAGGTATGCCT
GGACAAGCAATTGATGGTGTTCAGCTAAACTATATAACTCCTAAGGGAGAAAAATTATCACAGGCTTACTATCGTTCACAAA
CTACGAAACGATCAGGCTGGTTAAAAGTAAGTGCAGATAATGGTTCTATTCCTGGACTAGACAGTTATGCAGGAATCTTTGGA
GAACCGTTGGATCGCTTGCAAATAGGTATTTCACAGTCAAATCCATTTTAAATTTAGAAAATGACAGTAGGATTATGCAT

>Enterocin EJ97

AATTTTGTTGCCTTAAAAGGATTACAGCCAGAAAAAAAGAGATTCTAATATTGTTGCAATTAAAGATAGTAGGGTAACCTCTT
TTTTATTGTTCAAAAGAGGTTTAAATGAATAAAAATACCACAAGAATATTACGATCAATACTACAAATATAAAAATATAAAA
TTTAAACATTTGTGTTCACCCACTTTCAAAATTAATTATCAATATTTTAACAAAAATAGTAAAAATCTATTTAAAGTATGCTAA
AATGTAATTGTCTATATCTATTATAATCTTATAGGCATAACAATGAAAGGAGGAAAATTAATGTTAGCAAAAATTAAAGCGAT
GATTAAGAAATTCCCTAACCCTTATACTTTAGCAGCTAAGCTAACGACTTACGAAATTAATTGGTATAAACAACAATACGGTC
GTTATCCTTGGGAGCGCCCTGTAGCATAAAAAATATGAAGGGCAAGTAACTTCACTTGCCCTTCATATTTTTTATGCTACAGA
AAGAATAATATTACTAGGAGGATAGTATGCATAAGGAAAAATATAACTTATTTAATCTATTTATTGACAAGAAAACAATGTT
ATGCATTTTAATACCCTTACTAATAAGTATGGTTGCCGCAATCGTTTCTGTTCAAATTCCCCTCGTTTTAAAAAGAATTATCGA
TATATTGACAGATGGGAAACCATTGAATAACGAATTACTTTACCTATTAGTTATTTTTACTATTGGACAGCTTATCCTACAAGT
AATTTCTGATTTTTATCTACAAAAAATAGGAATAATTATTGTAAATAAAGTAAGAGCTAAAGTTATAAAACAAATACTAAATA
TGCGAACTTCTTTCTTTGAGCATTCTCTTTCTGGTAATATCGCTAGTATATTATCAAATGATGCAAGTAGTATGTATACTTTAGT
TAGTTCTACAATACCCAAAGTTGTGTTATCAATATTTGAAGTTCTTTTATATGGGATAGTCCTAATTAACCTTAGTGCAAAGTT
AACATTAGTAATTCTTGTTATAATACCATTAATATTTTTAATTTATTTACCTTTAGGTAGTTATATCGAGAAAAACTATTCAGA
AATGCAAAAAGAAATTGGTAATTTAAATGCTTTTGGAACGTTTATAACTAGAAGTCAGAAATATATAAAAATAAATAATACT
CAAAAAAAAGAAGAACAAAGCGGAAAACTTATATTATCCAAATTAAAGTATATAGGTTTAAAAAAGGCTAAACTAACTGCTA
TCATATCCCCTTTATTAGGAGCTTTATCACTATCAAGTATTGTATTTGTTGTTTTGCTGGGTTTTTATTTAATCTCATTAAAACA
ATTGACTACAGGTGCTTTAGTAGCATATTTGACATTATTTTTTCAGATAGTTACACCGATCGCTTCTATTGGTGAGTCATTCAC
AGAGTTTAAAGGCTTAATAGGAACTACTGAGAGGTTAAAACTATTACTAAAAAGTAATAATATTGAATCTTTATACGATGGTC
GAATGATACCTAACAATTCTATACAGAAAGTTGAGTTCAAGAACGTTAACTTTACTTATCCAGTTGAAGATAATATAAAAGAA
AAATCTTTTTCGTTAAAAGATATTTCTTTTAAAGCTAAAATTGGAGAAAATATCGCATTTGTCGGTCCTAGCGGTGCTGGTAA
AACTACTATATTCGATTTGTTAGAATGTTTTTACAGTATAGATTCTGGAGACATATATATGAACGACTATCATTATGACTATTA
TAATGTCTATAGTATCAGAAAAAATATTAGCTATGTTTCCCAATCATACCCACTTATAAACGGAACAATTTTAGAAAACTTAC
TATATGGGTTAGAAGAAAATATATCTGAACAGGAGATATCACTAGCTGCAAAGAAAACTAATTTTGACTTAGTTATTGATCGT
ATGCCTCAAGGATTTAATACTTATATTGGAGAAGAAGGTCAGCTTCTGTCTGGTGGAGAGAAACAAAGATTGGCATTAACTA
GAGCTTTTTTAAATCCAACATCATTAGTACTTTTGGATGAAGTCACATCAGCTTTAGATGCAGAAAATGAATACATCATTCAA
GAAAGTATTGTTTCATTAAAAAAAGAACGTATTATATTTACTATTGCTCATAGACTATCCACAATACAAAATTCTGATAAAAT
AATTTTTTTACAAGAAGGAAAAATTACAGGAATAGGCACACATGATGAGTTGTTGGAAAATCATAAATTATATAAAAAGTTT
ATTGACATTCAATTTAGAAAGTTTAGCTAAGAAATATATTGTTATAATACATTAATTTTTAAAAATTATAAAATATGGAGGTT
ATTATGGAAGTTTCATTAATATTTTTGCTTGGTATCTTTTGTGTATTCATTTTCTTTATAATTAAATTAATTAAGGAAAAACGTA
AAATTAATGTTGGAGAGATACTTTTTATTGTGATTTGTGTGTGTATAATAGTATTATTAGGAGTATTTTTAATTCGGGGAGATT
TTGTTGAAAGGCCAATCATTATATCACCAACTTAAATATTTTTGTATTTAGAAACTTCTTCTGTGCGTCAACAACCATGCATAG
CTATGCATAGTTGTTGGAATCATTGTAACATAAGTACAGAAGCTTGTTCTGGAGGAAGTAAATAAGGAAAAATAGTGCATTCT
ACAGTTGTTTCTTCTAATGTAGAACAGTCCAAGGAAACAGTAAAAAGTTAGCTTGACGAAGCAAAAGATAAATCCAATAAAG
CCATTGAGGGTGCTAGCAAAGTGTTAGAGTCTTCAGATATAAACAACTAACAAATATAAAATATCAGGATGATCCTATCATG
ACAGATAACGGAACAACAGATAAAATCAAAGGAAAAGCAAAAGTAGTTGCAGGAAATGTGACTGGGGATGATAAACAAAA
ATCAGAAGGTTTCTTAGATCAATCCATTGGCAAAGCAAAAGAAGTCGCTTCTGATGCTAAGGAGAAAGTCGAAGATGTTATT
GAAGATGTAAAAGAAAAATTGGATAAATAATTAAAAGAAAGGCAGATCCTTACGGT

>"Bacteriocin A"

CTAGCAAACTAGTTTTACCATTTTAGAGATAATTGCTTCTATAAATTTGAGTACCAAAGTAAAAATCCATGTTTCTGAAACGC
GTATAAATACTAGATATCAAACGTTTTGCACATGGATAGATAAACAAAGAGAGGAGGTGAAAAAATGAAGTTAAATAATGTA
TTATTTAATAAAAAAATGTATATGGGTGTAGCTTTTGTATTAGTAGCTTTTGCAATTAGTCTGACTCAACCACATTTAACATCA
ACATTAGGAATTTCAGCTTATGCAGCTAAAAAAGTAATTGATATTATTTCTGCTGCTAGTTCTGTTGCGGCTGTAGTAGGAAT
AATTGCAGCTGTTGTTGGTGGAGGTGGTATTGGTGTAGCAGTTCTAGCTACAGCAAAAGCTTTGGTAAAAAAATATGGAAAA
GCATATGCTGCTGCATGGTAAAAATTATATTATTAACGGACAGCTTTTAAAGCTGTCCGTTAATAATATAGGAGGTAATAACC
AATGGGAAATATTTATTTGAAAATTTTCAAAAGAAAATTAAGGATTGTAGAAAACTTTCACATTTTTAAATTATTTTTTTTAAT
CATGTATGTGTGTACGTGTACGTTATTATTATTAATAATCAATTTAAATAATTTGTTACTTCCTATTATCTTAATAAATTTTTTG
CTGTTAAACTATAATTTGCCAAAAATAGAGTTAGAGTTAAATCAAATGACAAGTTATAACTATTTTTACTCAGATAAGGTATT
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>"Bacteriocin B"

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>Cytolysin LS

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Appenddix 5: Protein sequences of the structural bacteriocin genes detected with BAGEL4

Enterocin Nkr-5-3B with a 62 % match in the structural protein found in genome P8-1:

```
Query              MKLNNVLFNKKMYMGVAFVLVAFAISLTQPHLTSTLGISAYAAKKVIDIISAASSVAAVVGIIAAVVGGGGIGVAVLATAKALVKKYGKAYAAAW
                                                P LT+ LGIS+YAAKKVIDII+  S+VA ++ ++ AVVGGG I   ++ATAK+L+KKYG  YAAAW
Enterocin_Nkr_5_3B          MKKNLLLVLPIVGIVGLFVGAPMLTANLGISSYAAKKVIDIINTGSAVATIIALVTAVVGGGLITAGIVATAKSLIKKYGAKYAAAW
```

Enterocin Nkr-5-3B with a 82 % match in the structural protein found in genome P8-1:

```
Query              MKKKLVKGLVICSMIGIGFTALGTNAEASARSYGNGVYCNKQKCWVNWNEAKQQIAGIVIGGWASSLSSMGR
                   MKKKLVKGLVIC MIGIGFTALGTN EA A  YGNGVYCNKQKCWV+W+ A+ +I
Enterocin_SE-K4    MKKKLVKGLVICGMIGIGFTALGTNVEA-ATYYGNGVYCNKQKCWVDWSRARSEIIDRGVKAYVNGFTKVLGGIGGR
```

Cytolysin ClyLS with a 98 % match in the structural protein found in genome P8-1:

```
Query              VLNKENQENYYSNKLELVGPSFEELSLEEMEAIQGSGDVQAETTPACFTIGLGVGALFSAKFC
                   +LNKENQENYYSNKLELVGPSFEELSLEEMEAIQGSGDVQAETTPACFTIGLGVGALFSAKFC
Cytolysin_ClyLs    MLNKENQENYYSNKLELVGPSFEELSLEEMEAIQGSGDVQAETTPACFTIGLGVGALFSAKFC
```

Cytolysin ClyLI with a 98 % match in the structural protein found in genome P8-1:

```
Query              MENLNVVPSFEELSVEEMEAIQGSGDVQAETTPVCAVAATAAASSAACGWVGGGIFTGVTVVVSLKHC
                   MENL+VVPSFEELSVEEMEAIQGSGDVQAETTPVCAVAATAAASSAACGWVGGGIFTGVTVVVSLKHC
Cytolysin_ClyLI    MENLSVVPSFEELSVEEMEAIQGSGDVQAETTPVCAVAATAAASSAACGWVGGGIFTGVTVVVSLKHC
```

Enterocin EJ97 was found in genome P8-1 with a 100 % match in the structural protein.

MKGGKLMLAKIKAMIKKFPNPYTLAAKLTTYEINWYKQQYGRYPWERPVA

Enterolysin A was found both in genome P7-17 and P6-20 with had a 100 % match in the structural protein.

MKNILLSILGVLSIVVSLAFSSYSVNAASNEWSWPLGKPYAGRYEEGQQFGNTAFNRGGTYFHDGFDFGSAIYGNGSVYAVHDGKILYAGWDPVGGGSLGAFIVLQAGNTNVIYQEFSRNVGDIKVSTGQTVKKGQLIGKFTSS
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LNYITPKGEKLSQAYYRSQTTKRSGWLKVSADNGSIPGLDSYAGIFGEPLDRLQIGISQSNPF

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