

DGGE analysis of probiotic-induced alteration in the canine faecal lactic acid bacteria microbiota

Susanna Peiponen

Advanced Studies

University of Helsinki

The Faculty of Veterinary Medicine

Department of Equine and Small Animal Medicine

Research mentors: DVM, PhD, Adjunct Professor Minna Rinkinen

PhD Shea Beasley

MSc Titta Manninen

2008



Tiedekunta – Fakultet - Faculty Faculty of Veterinary Medicine		Laitos – Institution – Department Department of Equine and Small Animal Medicine	
Tekijä - Författare – Author Susanna Peiponen			
Työn nimi – Arbetets title – Title DGGE analysis of probiotic-induced alteration in the canine faecal lactic acid bacteria microbiota			
Oppiaine – Läroämne - Subject Internal Medicine			
Työn laji – Arbetets art - Level Advanced Studies	Aika – Datum – Month and year March 2005 - May 2008	Sivumäärä – Sidoantal – Number of pages 27	
Tiivistelmä – Referat -Abstract <p>Several canine clinical gastrointestinal problems, such as inflammatory bowel disease and small-intestinal bacterial overgrowth, are believed to be associated with bacteria. These conditions often lead to chronic diarrhea. Many canine gastrointestinal problems are treated with antibiotics, but due to increasing problems with antimicrobial resistance alternative therapies should be considered. One of these therapies is suggested be probiotic bacteria treatment, especially with members of the genus <i>Lactobacillus</i> due to their health-conferring properties.</p> <p>The probiotic characteristics of bacteria are linked to host specificity which is an important criterion for selection of a probiotic. Most of the commercial probiotic strains meant for dogs are not from canine origin. In addition many substances available in the Finnish market contain <i>Enterococcus faecium</i>, whose safety has been questioned due to it's pathogenic characteristics.</p> <p>This study was based on the study project investigating the effect of host-specific canine LAB on long term gastro-intestinal symptoms. The study was a randomized, double-blinded and placebo controlled trial. This study examined the changes in the canine faecal LAB microbiota caused by fed canine host-specific probiotic LAB. The purpose was to become familiar to the isolation of gastro-intestinal microbiota and to assess the potential alterations in the faeces by applicable methods. The most important methods were polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE).</p> <p>During the study DGGE analysis proved to be a working method for analyzing bacterial communities. When the method becomes more familiar, it will probably be as common in use as agarose gel electrophoresis due to it's advantages. Some alterations of the microbiota could be seen on the DGGE analysis. During the experiment the bands resembling those of standard strains were dominant. This implies the <i>Lactobacillus</i> strains of interest being able to survive the canine intestine. However, after feeding seized the bands formed by the strains of interest disappeared and were replaced with bands of indigenous LAB. Also after the feeding seized, some new bands appeared on DGGE gel. This implies a contribution of fed probiotic mixture to the enhanced prevalence of potential novel microbes.</p>			
Avainsanat – Nyckelpord –Keywords lactic acid bacteria, canine intestine, DGGE, PCR specific primers			
Säilytyspaikka – Förvaringställe – Where deposited Viikki Science Library			
Muita tietoja – Övriga uppgifter – Additional information Research mentors: DVM, PhD, Adjunct Professor Minna Rinkinen, PhD Shea Beasley, MSc Titta Manninen			

Abbreviations

AGE	agarose gel electrophoresis
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EtBr	ethidium bromide
GI	gastrointestinal
LAB	lactic acid bacteria
LAB8	<i>Lactobacillus fermentum</i> strain LAB8
LAB9	<i>Lactobacillus salivarius</i> strain LAB9
LAB11	<i>Lactobacillus rhamnosus</i> strain LAB11
LAB12	<i>Lactobacillus mucosae</i> strain LAB12
PCR	polymerase chain reaction
RNA	ribonucleic acid
16S RNA	ribosomal RNA

Contents

1. Introduction	4
1.1. Lactic acid bacteria.....	4
1.2. Identification of LAB.....	5
1.3. The use of LAB as probiotics.....	6
1.4. Aim of the study.....	7
2. Materials and methods.....	8
2.1. Project	8
2.2. Study participants	8
2.3. Supplementation	8
2.4. Faeces collection and reporting.....	9
2.5. Isolation of DNA.....	9
2.6. PCR.....	10
2.7. AGE.....	11
2.8. DGGE	12
2.9. Implementation of the present study protocol.....	13
2.9.1 Isolation of DNA from faeces	13
2.9.2 AGE	14
2.9.3 Isolation of DNA from plated faeces	14
2.9.4 PCR.....	15
2.9.5 DGGE.....	16
3. Results	18
4. Discussion	21
5. Conclusions.....	23
6. References.....	24

1. Introduction

Several canine clinical gastrointestinal problems, such as inflammatory bowel disease and small-intestinal bacterial overgrowth, are believed to be associated with bacteria (Shanahan 2004). These conditions often lead to chronic diarrhoea. Many canine gastrointestinal problems are treated with antibiotics, but due to increasing problems with antimicrobial resistance, alternative therapies should be considered (Shanahan 2004). One of these therapies is suggested be probiotic bacteria treatment, especially with members of the genus *Lactobacillus* due to their health-conferring properties (Walter et al. 2000).

Traditional cultivation and characterization methods have been applied to many bacterial studies, hence potentially biased the identification and taxonomy of the intestinal microbiota due to unculturable bacteria (Rinkinen 2006). Bacterial taxonomy and nomenclature have also changed during time, so the bacteria identified earlier may now be re-classified as under a different name (Rinkinen 2006). Cultivation is however still widely used due to its advantages, i.e. low cost and simplicity.

1.1. Lactic acid bacteria

Lactic acid bacteria (LAB) form a heterogeneous group of gram-positive bacteria. The bacteria included in the group are gram-positive, nonsporing, nonrespiring cocci or rod, which produce lactic acid as the major end product during the fermentation of carbohydrates (Axelsson 2004). The best known genera are *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Weissella* (Rinkinen 2004, p.13). *Lactobacillus* is the largest of the genera included in the LAB (Axelsson 2004).

LAB can be found in food, fermented products, milk, soil, water, manure, sewage, animals and humans. LAB can also be a spoilage organism in food and beverages (Liu 2003).

Host-associated bacteria with their metabolic contributions to host physiology have clear trophic functions and play a role in protecting the host against pathogenic species (Mai and Morris, 2003). LAB comprise an important part of the human and animal intestinal microbiota. Although LAB are known to perform an important role in the intestine, there are only few reports on the role of these bacterial species in canine intestine (Benno et al. 1992, Davis et al. 1977, Goldin and Gorbach 1984, Greetham et al. 2002). Most of the canine intestinal LAB belong to the genera *Streptococcus* and *Lactobacillus* (Rinkinen 2006).

1.2. Identification of LAB

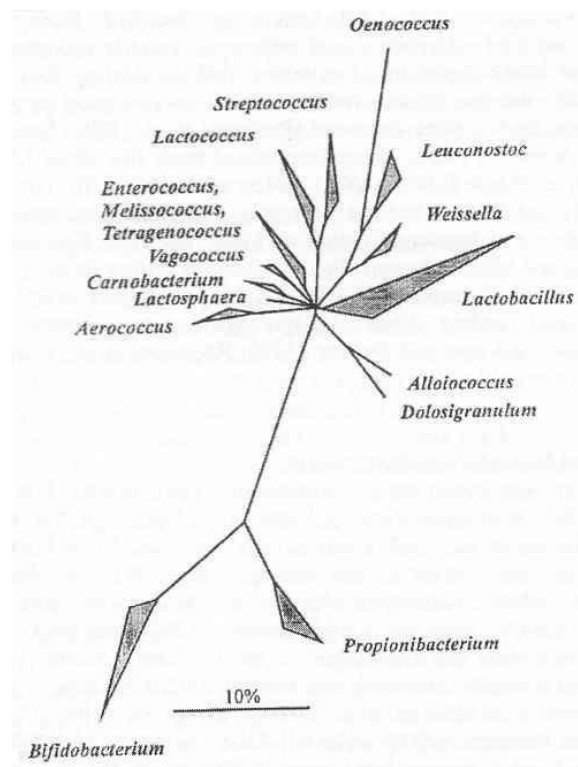
LAB can be divided into rods (*Lactobacillus* and *Carnobacterium*) and cocci (all other genera) (Axelsson 2004). An important characteristic used in differentiation of the LAB genera is the mode of glucose fermentation under standard conditions (Axelsson 2004). LAB can use two different main sugar fermentation pathways. Homofermentative bacteria yield two lactates from one glucose and heterofermentative transform a glucose molecule into lactate, ethanol and carbon dioxide (Axelsson 2004).

The classification of LAB at species level currently relies on molecular biology methods. The genus *Lactobacillus* consists alone about 80 recognized species. (Axelsson 2004)

The use of comparative sequence analysis of the 16S ribosomal ribonucleic acid (rRNA) gene has become a very important tool in classifying microorganism as it reflects the natural evolutionary relationships or phylogeny (Vaughan et al. 2002). It is regarded to be the optimal measure for determining true phylogenetic relations among bacteria (Axelsson 2004). When comparing the 16S rRNA sequences of two different organisms they can be regarded as same genus when the sequence > 97% similar. Deoxyribonucleic acid (DNA)-hybridization can be used for determining relationships to species level. When the DNA hybridizes >70% they can be regarded as the same species (Brock et al. 1994).

The taxonomy of the LAB based on 16S rRNA has presented challenges studying and identifying the intestinal tract LAB but various molecular approaches have been developed (Vaughan 2002). These approaches consist of several techniques, such as polymerase chain reaction (PCR) based methods, denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) (Zoetendal et al. 2004).

The consensus tree of the LAB can be seen in the picture 1. The scale below the tree shows the distance between two genera that differ 10% in the comparison of 16S rRNA.



Picture 1. The consensus tree of major phylogenetic groups of lactic acid bacteria based on the comparative analysis of the 16S rRNA gene. The tree includes also the nonrelated Gram-positive genera *Bifidobacterium* and *Propionibacterium*. (Holzapfel et al. 2001)

1.3. The use of LAB as probiotics

Probiotics are living microbes, which are used to promote health as such or as a food product (Saxelin 2002). These microbes usually belong to the species *Lactobacillus* sp. or *Bifidobacterium* sp. (Chang et al. 2001, Saarela et al. 2000). The

ingestion of probiotic LAB has many documented or potential benefits, such as modulation of the GI-tract, antagonism against pathogenic microbes, and maintaining the intestinal mucosal barrier (Ouwehand et al. 2002, Saarela et al. 2000). Adhesion to the intestinal mucosa is considered to be one of the main mechanisms for the probiotic LAB to benefit the health of the host (Rinkinen 2004 p.11). Some strains of LAB have been documented to have beneficial effects on the health of dogs (Baillon et al. 2004, Benyacoub et al. 2003).

The probiotic characteristics of bacteria are linked to host specificity which is an important criterion for selection of a probiotic (Ouwehand et al. 2002). Other criteria are bile and acid tolerance and survival in the GI-tract (Saarela et al. 2000).

Most of the commercial probiotic strains meant for dogs are not from canine origin (Beasley 2004, Manninen et al. 2006). In addition, many substances available in the Finnish market contain *Enterococcus faecium*, i.e. Tehobakt, Aptus, Orionpharma and Biobak, Biofarm (Anonymous a, Anonymous b), whose safety has been questioned due to its pathogenic characteristics (Rinkinen et al. 2003).

1.4. Aim of the study

The aim of the present study was to examine the changes in the canine faecal LAB microbiota caused by fed canine host-specific probiotic LAB. The purpose was to become familiar to the isolation of gastro-intestinal microbiota and to assess the potential alterations in the faeces by applicable methods.

2. Materials and methods

2.1. Project

This report is based on the study project investigating the effect of host-specific canine LAB on long term gastro-intestinal symptoms using DGGE. The study was a randomized, double-blinded and placebo controlled trial. The most important methods are PCR and DGGE.

The members of the project group were DVM, PhD, Adjunct Professor Minna Rinkinen, Professor Per Saris, MSc Titta Manninen, PhD Shea Beasley, CVM Aija Mehtälä and CVM Susanna Peiponen. The laboratory work for the current study was completed in the laboratory of professor Per Saris at the University of Helsinki Faculty of Agriculture and Forestry Department of Applied Chemistry and Microbiology. The study was funded by the Academy of Finland (project number 177321). The study had a recommendation of the Test Animal Committee of the University of Helsinki. Responsibilities in the project were divided amongst the team members so that Dr Rinkinen with CVM Mehtälä were responsible for the statistical processing of the report forms, whereas Professor Saris, MSc Manninen and CVM Peiponen were responsible for the microbiological cultivation and molecule biological methods.

2.2. Study participants

Dog owners with pets suffering from chronic diarrhoea were requested to take part into the project. The project initiated with a total of 56 participating dogs throughout Finland. The dogs were selected according to their gastro-intestinal problems, i.e. diarrhoea and vomiting.

2.3. Supplementation

Half of the participating dogs received 5×10^9 cfu/g of each lactic acid bacteria (*Lactobacillus fermentum* LAB8, *Lactobacillus salivarius* LAB9 and *Lactobacillus rhamnosus* LAB11) mixed in 1g CaCO₃ for three months. CaCO₃ served as a placebo (1 g) for the rest of the participants.

2.4. Faeces collection and reporting

The pet owners were requested to report changes in the dog's intestinal problems by the means of a questionnaire. In addition, faeces from the dogs participating in Helsinki region were collected in order to identify the effect of the fed substance on intestinal microbiota. The collected faecal amount was 2-18g per dog.

2.5. Isolation of DNA

The first step in analyzing DNA is to isolate chromosomal DNA. There are basically three stages: cellular lysis, removing other components than DNA, and DNA concentration (Oksanen 2002).

The cellular lysis can be done either chemically or manually. When breaking down the lipid layer of an animal or bacterial cell, the lysis is usually done chemically. Lysis can be performed either enzymatically with lysozyme or with chemical detergents such as SDS or EDTA. When using detergents proteinase K can be additionally used to break down proteins. (Brown 2006)

After the cellular lysis the mixture contains DNA, RNA and proteins. Proteins are separated from the mixture with chloroform and isoamyl alcohol. The mixture forms two layers, the lower organic phase and the upper aqueous phase containing the DNA material. Most of the proteins denature and form a white layer between the two phases. After this the DNA can be collected from the mixture by removing the aqueous phase into a clean test tube.

The DNA is precipitated with either isoamyl alcohol or ethanol (Suominen and Ollikka 2003). DNA forms a salt and can be centrifuged to form a pellet.

The excess liquid can be removed and the DNA pellet resuspended into a buffer solution or sterile water.

2.6. PCR

PCR is a way to multiply a desired DNA sequence. The reactions take place in a temperature-controlled environment. The main key is to use thermostable DNA-polymerase, which doesn't inactivate in high temperatures. These polymerases have been isolated for example from bacteria living in hot springs (Suominen and Ollikka 2003).

The DNA sequence of interest works as a template. The DNA sequence needs to locate between two known sequences in order to get primers to anneal to the DNA. The primers anneal at the opposite ends of DNA of interest. The polymerase then works between the primers.

The PCR reaction holds three basic steps (Picture 2).

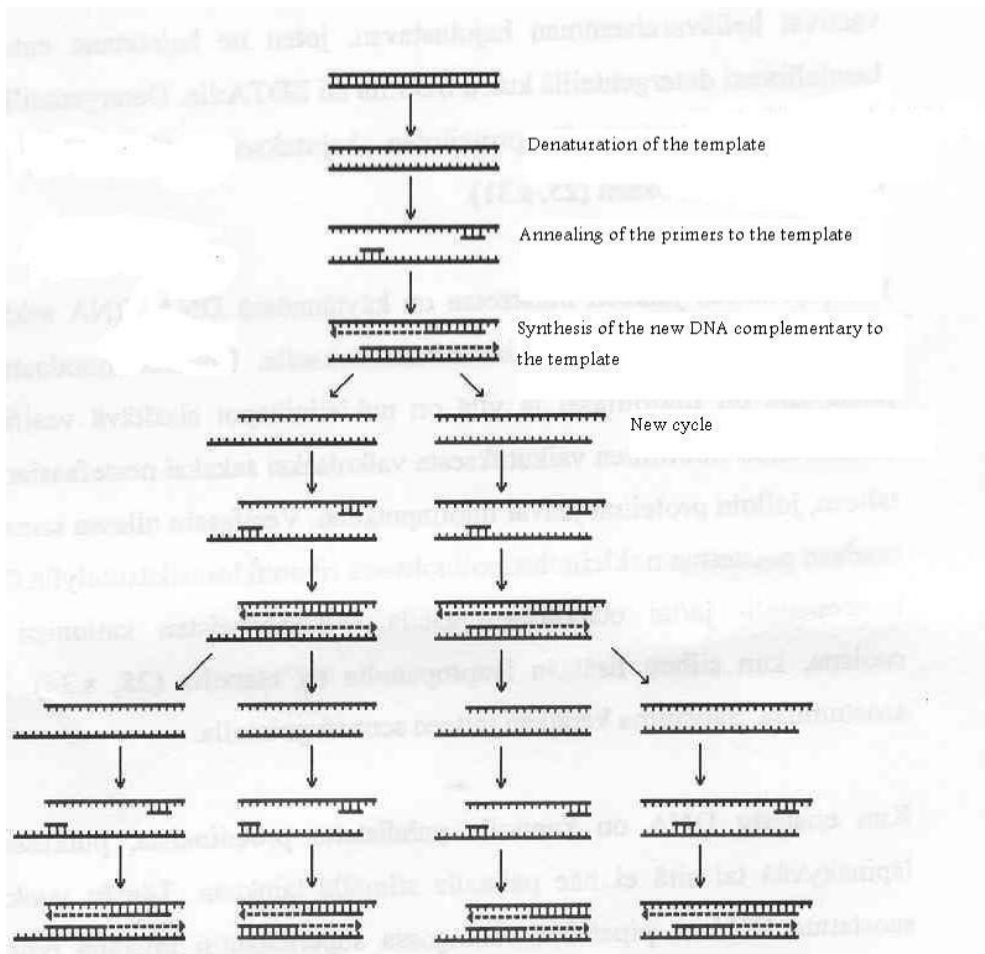
First the mixture is heated to a temperature of 94 °C. This allows the hydrogen bonds holding the double-stranded DNA together to break (Brown 2006). This is called denaturation.

The mixture is then cooled down to 50-60°C. At this point the primers attach to the template, but the template doesn't have enough time to renature (Suominen and Ollikka 2003).

The temperature is raised to about 74 °C. This is optimal temperature for the DNA polymerase to work. The polymerase attaches beside the primer and starts to make new DNA from the nucleotides added to the mixture. The DNA is complementary to the template (Brown 2006).

After this step the temperature is raised back to 94 °C and the cycle is repeated for 15-40 times.

If the reaction works optimally the amount of DNA can be amplified 10 000 times from the amount in the beginning (Oksanen 2002).



Picture 2. Polymerase Chain Reaction (Oksanen 2002).

2.7. AGE

There are many different ways to analyze DNA which based on electrophoresis. AGE (agarose gel electrophoresis) is used to analyze medium-sized 0.1 – 50 kb DNA fragments (Suominen and Ollikka 2003).

The method is based on the negative charge of DNA molecules. When molecules are placed on an electric field, the molecules start to migrate towards positive pole called anode (Brown 2006).

The electrophoresis is located on an agarose gel. Agarose is a polysaccharide isolated from seaweed, which forms a gel after it has been boiled (Suominen and Ollikka 2003). Agarose is used to slow down the migration of the fragments. The DNA samples are placed on multiple little wells on the gel. Then the power is turned on and the fragments start to move. Fragments with the same size migrate with the

same speed through the mesh of agarose, and therefore gel electrophoresis can be used to separate DNA according to their size.

Fragments of the same size form bands on the gel, which can be visualized with ethidium bromide (EtBr) and ultraviolet light. Ethidium bromide infiltrates between the bases on the DNA. When the mixture is placed under uv-irradiation, the ethidium-DNA complex fluoresces (Suominen and Ollikka 2003). The problem with EtBr is that it is highly mutagenic and should be handled with caution (Brown 2006).

2.8. DGGE

DGGE is a method to separate DNA fragments of the same length but with different sequences (Muyzer and Smalla 1998). DGGE can be used for direct analysis of genomic DNA from organisms or PCR can be used to selectively amplify the sequence of interest before DGGE is used if the sequence of interest is known (Cariello et al. 1988). DGGE can be used for whole community analysis, or for studying specific populations or groups within the sample (McCartney 2002).

Separation in DGGE is based on the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule. The melting is caused by polyacrylamide gel containing a linearly increasing gradient of denaturants, mixture of urea and formamide. (Muyzer and Smalla 1998)

During DGGE, the DNA fragments encounter increasingly higher concentrations of chemical denaturant as they migrate through the polyacrylamide gel. Upon reaching a threshold denaturant concentration, the weaker melting domains, stretches of base pairs with similar melting temperatures of the double-stranded DNA will begin to denature at which time migration slows dramatically (Anonymous 2004 c).

To increase the detection of different sequence variations GC-rich sequences, so-called GC-clamps, can be incorporated into one of the primers. The GC-clamp acts as a high melting domain preventing the double-stranded DNA to dissolve

completely and thus migrating off the polyacrylamide gel. (Muyzer and Smalla 1998)

The bands of DNA of interest can be visualized using ethidium bromide, as in AGE.

PCR-DGGE has been used for detecting different *Lactobacillus*- species of gastrointestinal origin (Endo et al. 2007, Sanz et al. 2007, Walter et al. 2000, Wang et al. 2007).

2.9. Implementation of the present study protocol

2.9.1 Isolation of DNA from faeces

DNA was isolated from 0,5 g of each dog faeces. The sample was suspended on 15 ml of wash buffer (50 mM NaPO₄ , 0,1% Tween 80, pH 8). The suspension was then shaken horizontally 150 rpm for 15-20 minutes, centrifuged (Beckman, Palo Alto, CA, USA) low speed 200-300 rpm for 15-20 minutes. The aim of this stage is to remove the faeces particles from the supernatant. After removing the additional particles the supernatant was centrifuged for 15000 rpm for 15 minutes. The pellet was resuspended in wash buffer and centrifuged again for 15000 rpm for 15 minutes.

The pellet was resuspended into 1.5 ml of TE buffer (10 mM Tris, 1 mM EDTA). The suspension was then subjected to four rounds of freezing (-70°C 1 h) and thawing (+37°C 30 hours). On the fifth round the suspension was kept in -70°C for overnight and in +37°C for 30 minutes. The main goal for this freezing and thawing is to accomplish bacterial lysis.

After the suspension was thawed 0.35 ml of lysozyme (200 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) was added to the suspension and the mixture was incubated for 2-3 hours in +37°C. 10 µl of proteinase K (20 mg/ml, Finnzymes, Espoo, Finland) and 0.14 ml of sodium dodecyl sulfate (20%) were added and the mixture was incubated +37°C for 1 h. Following this incubation 0.35 ml of 5M NaCl, 0.3 ml of 10% CTAB (hexadecyltrimethyl ammonium bromide) in 0,7M NaCl and 0.5 g of

1,000µm diameter glass beads were added, followed by 20 minutes of incubation in +65°C with vortexing every 5 minutes for 30 seconds.

Mixture was then added to an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 6000xg for 10 minutes at room temperature to separate the aqueous and organic phases. The upper aqueous phase was transferred into a clean test tube.

The DNA precipitated by adding 0.6 volume of isopropanol followed by 1 h incubation at room temperature. The DNA was collected by centrifugation at 15000 rpm for 15 minutes. The DNA pellet was finally washed with 1 ml of cold -20°C 70% ethanol and centrifuged at 15000 rpm. The DNA pellet was finally resuspended into 50-200 µl 1x TE buffer. (Apajalahti et al. 1998)

2.9.2 AGE

The AGE gel contained 1.6g of agarose, 200 ml of 1x TBE-buffer and 10 µl of EtBr. The final gel concentration was 0.8%.

5 µl of each sample was added into well with 1 µl of loading dye. The mass standard wells contained 1 µl of mass standard, 4 µl of double deionised water and 1 µl of loading dye summing up to 5 µl. The ladder wells contained 5 µl of 1 kB ladder solution.

The voltage used was 120 V for 60 minutes.

2.9.3 Isolation of DNA from plated faeces

The problem with PCR and samples collected from faeces is that faeces contain a large amount of PCR inhibitors (Chambers et al. 2001). In this study the problem of large amounts of PCR inhibitors was avoided by first plating the faeces to *Lactobacillus* selective mLBS-medium (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) and growing the pool overnight for three days at +37°C. LBS broth (2 ml) was added on top of the 10⁻² dilution plates. Suspension was collected, mixed with 0.5 ml of sterile 87% glycerol and frozen at -20°C.

After this the isolation of DNA was done from 2 ml bacterial suspension as described below in picture 3. by Anderson and McKay (1983). In addition to the method by Anderson and McKay (1983), the samples were dipped into liquid nitrogen before adding lysozyme (Sigma-Aldrich, St. Louis, MO, USA). The lysozyme (100 mg/ml) was employed with 20 mg/ml proteinase K (Finnzymes, Espoo, Finland) for 1 h at 37°C. RNase (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added at the end of the isolation procedure.

Step	Details of following protocol:	
	Screening (1.5–10 ml) ^a	Preparative (600 ml) ^a
Resuspend pelleted cells in 6.7% sucrose–50 mM Tris–1 mM EDTA, pH 8.0	379 µl	30 ml
Warm to 37°C		
Add lysozyme (10 mg/ml in 25 mM Tris, pH 8.0)	96.5 µl	7.5 ml
Incubate for 5 min at 37°C		
Add 0.25 M EDTA–50 mM Tris, pH 8.0	48.2 µl	3.75 ml
Add sodium dodecyl sulfate (20% [wt/vol] in 50 mM Tris–20 mM EDTA, pH 8.0)	27.6 µl	2.25 ml
Mix immediately		
Incubate for 5 to 10 min at 37°C to complete lysis		
Vortex at highest setting for 30 s in an appropriate tube	1.5-ml Eppendorf	15 ml per tube (25 by 150 mm)
Add fresh 3.0 N NaOH	27.6 µl	2.40 ml
Mix gently by intermittent inversion or swirling for 10 min	Inversion	Swirl in 250-ml centrifuge bottle
Add 2.0 M Tris-hydrochloride, pH 7.0	49.6 µl	3.90 ml
Continue gentle mixing for 3 min		
Add 5.0 M NaCl	71.7 µl	5.7 ml
Add phenol saturated with 3% NaCl; mix thoroughly	700 µl	55.8 ml
Centrifuge	.5 min	5,000 rpm in GSA rotor, 10 min
Remove upper phase and extract with chloroform-isoamyl alcohol (24:1)	700 µl	55.8 ml
Remove upper phase, precipitate with 1 vol of isopropanol		
Incubate at 0°C	>30 min	>60 min
Centrifuge	.5 min	8,000 rpm in GSA rotor, 20 min
Remove excess isopropanol and resuspend in 10 mM Tris–1 mM EDTA, pH 7.5	20 µl	1,200 µl
Examine 5 to 10 µl by agarose gel electrophoresis		

^a The culture volume used in each protocol is indicated in parentheses.

Picture 3. Isolation of DNA from bacterial suspension (Anderson and McKay 1983).

2.9.4 PCR

Universal primers R1401 (5'-CGG TGT GTA CAA GAC CC-3') and F968-GC (5'-GC-clamp AAC GCG AAG AAC CTT AC-3') were employed. The universal primer F968GC contains a GC-clamp (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3') (Nübel et al. 1996, Zoetendal et al. 2002).

The PCR mixture contained 2 µl of the template and 23 µl of Master mix.

Master mix (1X) contained 15.8 µl sterile water, 5M 5 µl betaine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 10X 2.5 µl Dynazyme buffer (Finnzymes, Espoo, Finland), 0.5 µl dNTP (the nucleotides necessary for the reaction, Finnzymes, Espoo, Finland), 0.5 µl x2 of both primers (Oligomer, Espoo, Finland), and 0.18 µl Dynazyme (DNA polymerase, Finnzymes, Espoo, Finland).

The universal primer PCR program was 94°C for 3 minutes (denaturing temperature), followed by for 35 cycles of 94°C for 1 minute, 51°C 1 minute (annealing temperature) and 72°C for 2 minutes (temperature optimal for DNA polymerase). A cycle of 72°C for 10 minutes ended the program (Table 1).

Table 1. The Polymerase Chain Reaction amplification program. The phases highlighted were repeated for 35 times.

Phase	Temperature	Time
Denaturing	94°C	3 minutes
Denaturing	94°C	1 minute
Annealing	51°C	1 minute
Synthesis of new DNA	72°C	2 minutes
Synthesis of new DNA	72°C	10 minutes
Storage	4°C	hold

2.9.5 DGGE

The DGGE analysis was conducted of the amplified canine faeces samples.

The DGGE gel wells were filled with 20µl of PCR mixture and 10µl of 2x loading dye.

The DGGE gel used in the project had the concentration of 35%-55% of urea and formamide from Bio-Rad DCode Electrophoresis Reagent Kit for DGGE and CDGE.

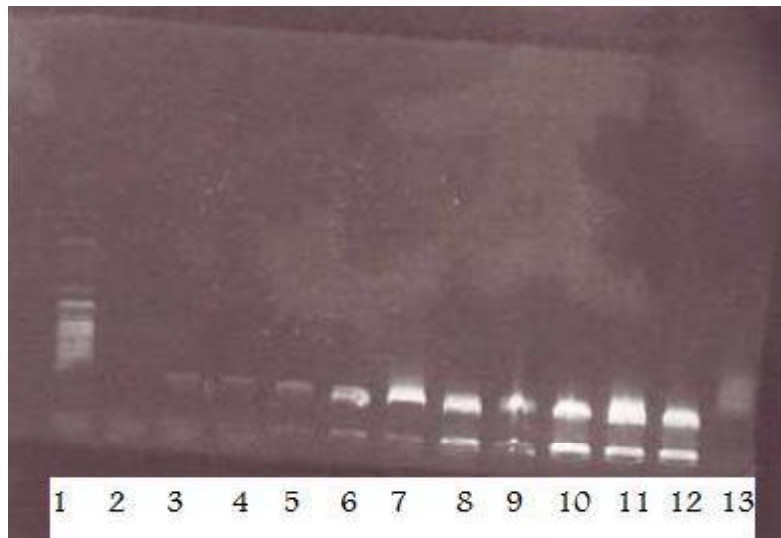
Solution with the low concentration 35% was made by adding 4,55 ml of 100% solution (15 ml 40% acrylamide/bis, 2 ml 50x TAE Bio-Rad, 40 ml formamide, 42g urea) and 8.45 ml of 0% solution (15 ml acrylamide/bis, 2 ml 50x TAE Bio-Rad, 83 ml water).

Solution with the high concentration 55% was made by adding 7,15 ml 100% solution, 5.85 ml of 0% solution and 60 µl of GelStar© nucleic acid stain (FMC BioProducts, Rockland, ME).

The temperature used was 60°C, time 4.5 hours and the voltage 150V in a Dcode apparatus (Bio-Rad, Hercules, CA, USA) containing a magnetic stirrer.

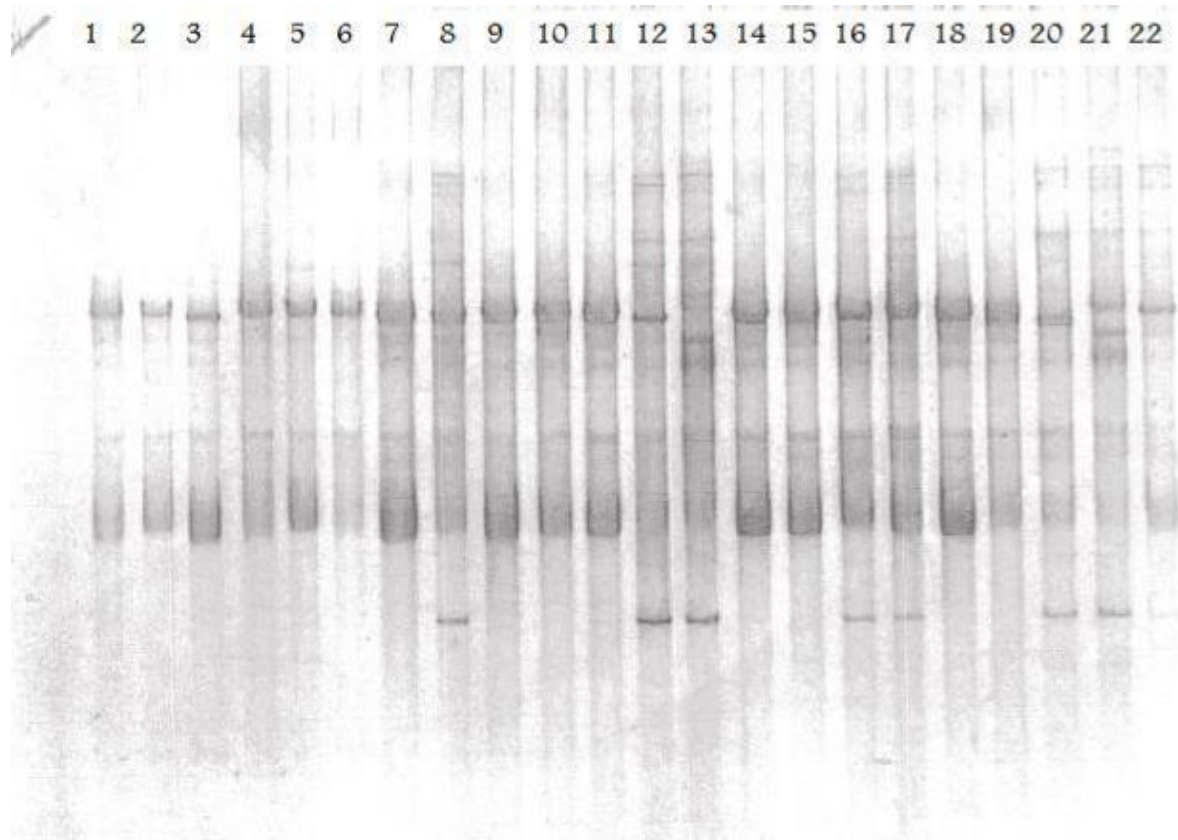
3. Results

The AGE done after the DNA isolation contained large amount of DNA, as seen in the picture 4.

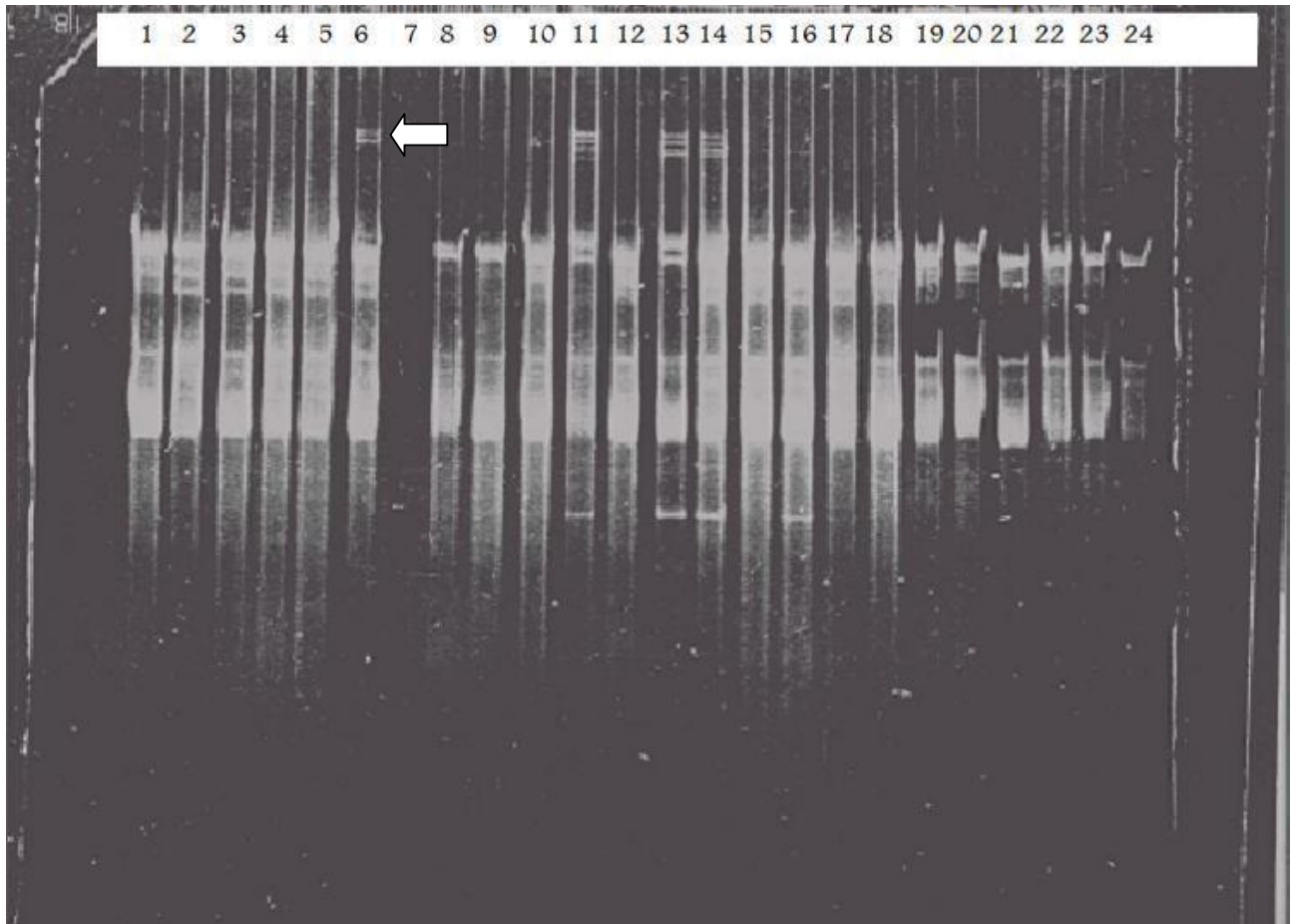


Picture 4. AGE after isolation of DNA. The lanes from left to right: 1. 1 kB ladder standard, 2. 20 mass standard, 3. 40 mass standard, 4. 60 mass standard, 5. 80 mass standard, 6. 100 mass standard, 7. sample from dog 44288 after one week, 8. sample from dog 76535 after one week, 9. sample from dog 10975 after two weeks, 10. sample from dog 98336 after two weeks, 11. sample from dog 48111 after three weeks, 12. sample from dog 90360 after three weeks, 13. 1 kB ladder.

In DGGE (Pictures 5. and 6.) the lanes were arranged so that samples from the same dog were side by side. During the experiment bands correlating with LAB standards are dominant. In most dogs the bands seen in the picture are different before and after the feeding than during the experiment. The canine microbiota reverted to the prior feeding situation when probiotic intake ceased. In one of the dogs new bands appeared on the DGGE gel after the feeding ended. These bands are of unknown bacteria, nor resembling the bands of the *Lactobacillus* -strains.



Picture 5. DGGE. Lanes from left to right: 1. LAB8 isolated from the substance fed to dogs, 2. LAB9 isolated from the substance fed to dogs, 3. LAB11 isolated from the substance fed to dogs, 4. LAB8 pure culture, 5. LAB9 pure culture, 6. LAB11 pure culture, 7. standard, all the LAB strains from pure cultures, 8. sample from dog 78925 before feeding of the substance, 9. sample from dog 78925 after 1 month, 10. sample from dog 78925 after 2 months, 11. sample from dog 78925 after 3 months, 12. sample from dog 78925 2 weeks after the feeding had ended, 13. sample from dog 44288 before feeding of the substance, 14. sample from dog 44288 after 1 month, 15. sample from dog 44288 after 2 months, 16. sample from dog 44288 after 3 months, 17. sample from dog 44288 2 weeks after the feeding had ended, 18. standard with all the LAB strains, 19. sample from dog 58209 before feeding of the substance, 20. sample from dog 58209 after 2 months, 21. sample from dog 58209 after 3 months, 22. sample from dog 58209 2 weeks after the feeding had ended.



Picture 6. DGGE. Lanes from left to right: 1. standard with all the LAB strains, 2. sample from dog 14684 before the feeding of the substance, 3. sample from dog 14684 after 1 month, 4. sample from dog 14684 after 2 months, 5. sample from dog 14684 after 3 months, 6. sample from dog 14684 2 weeks after the feeding had ended, new bands pointed with arrow, 7. sample from dog 07446 before the feeding started (sample propably not in the well) 8. sample from dog 07446 after 1 month, 9. sample from dog 07446 after 2 months, 10. sample from dog 07446 after 3 months, 11. sample from dog 07446 2 weeks after the feeding had ended, 12. standard with all the LAB strains, 13. sample from dog 00056 before the feeding of the substance , 14. sample from dog 00056 after 1 month, 15. sample from dog 00056 after 2 months, 16. sample from t dog 00056 after 3 months, 17. sample from dog 00056 2 weeks after the feeding had ended, 18. standard with all the LAB strains from pure culture, 19. LAB8 pure culture, 20. LAB9 pure culture, 21. LAB11 pure culture, 22. LAB8 isolated from the substance, 23. LAB9 isolated form the substance, 24. LAB11 isolated from the substance

4. Discussion

This study examined the changes in the canine faecal LAB microbiota caused by fed canine host-specific probiotic LAB. The purpose was to become familiar to the isolation of gastro-intestinal microbiota and to assess the potential alterations in the faeces by applicable methods.

To summarize the results, the method for isolation of the LAB DNA from the faeces described by Apajalahti et al. (1998) was applicable for this study. Strength of this method compared to other methods is the efficiencies of bacterial extraction and lysis (>95% and <99% respectively) and therefore the DNA recovered should accurately reflect the bacterial communities of the faeces (Apajalahti et al. 1998). After testing several methods for isolating DNA from faeces, the method described by Apajalahti et al. (1998) worked best.

In the DGGE analysis the bands resembled those of the LAB strains fed to the dogs formed on the DGGE-gel. DGGE analysis is a reliable way to analyze bacterial communities. However, in this study the reading of the DGGE gel was challenging due to maybe a bit too wide concentration gradient. For additional research smaller concentration gradient on the gel is suggested. At this study the results seen on DGGE gel are not as reliable as hoped.

Some alterations of the microbiota could be seen on the DGGE gel. During the experiment the bands resembling those of standard strains are dominant. This implies the *Lactobacillus* strains of interest being able to survive the canine intestine. However, after feeding ceased the bands formed by the strains of interest disappeared and were replaced with bands of indigenous LAB. This implies that the strains can't keep their status as a dominant LAB without dietary supplementation. This is in accordance of previously reports documenting that it is virtually impossible to change the intestinal microbiota permanently once it has established (Baillon 2004).

Interestingly, after the feeding ceased, some new bands appeared on DGGE gel. In order to identify the new bands and potential novel microbes the bands could have

been cut out from the gel and sequenced. This was not done in the present study. However, Manninen et al (2006) reported a similar outcome when the probiotic mixture was fed to laboratory beagles, documenting a contribution of LAB8 to LAB12 to the enhanced prevalence of *Lactobacillus acidophilus*. In their study the changes in the gut microbiota prevailed for a relatively long period. In the present study the length of the microbial alteration was not examined.

Strength of the study was finding an applicable method for analyzing bacterial community and alteration without cultivation. As the rapid molecular techniques evolve, they compensate to the traditional methods including bacterial cultivation. It is commonly recognised that some aspects of phenotypic characterization are principally flawed, i.e. observation of a similar phenotype does not always equate to a similar or closely related genotype (McCartney 2002).

Never the less, there were some limitations to the study: Isolating DNA from faeces would have given a wider knowledge of the existing microbiota compared to the use of cultured microbiota. It is estimated that only 40% of human intestinal microbiota is culturable, similar outcome can be expected also in dogs (Rinkinen 2006). DGGE analysis from faecal DNA would have detected also the microbiota that can not be cultivated. Also the DGGE- gradient used in the study was not accurate enough. At this study the LAB strains could not be identified from each other because the bands they formed in DGGE gel were so close to one another. Also the study turned out to be more challenging than expected. Finding applicable methods for this study took a lot of research. At the time of our study DGGE analysis was still a new technique. Also faeces still seem to be a challenging material for DNA isolation. It took many tries to find a successful method to isolate DNA.

5. Conclusions

During the study DGGE analysis proved to be a working method for analyzing bacterial communities. When the method comes more familiar, it will probably come as common in use as AGE due to its advantages.

Currently, the material costs of DGGE compared to AGE may limit the use of DGGE in everyday laboratory work.

More research is needed to determine the potential use of the used *Lactobacillus* strains as a probiotic, i.e. a clinical trial with documented effects on the GI-symptoms of the dogs and a trial focusing on the suggested selective advantage of the indigenous LAB resulting from the probiotic LAB strain supplementation is warranted.

6. References

- Anderson D.G., McKay L.L., Simple and Rapid Method for Isolating Large Plasmid DNA from Lactic Streptococci. Applied and Environmental Microbiology, September 1983, p. 549-552
- Anonymous a, Aptus® Tehobakt, OrionPharma, referenced 27.4.2008
<http://www.aptuspet.com/tuotteet?productgroup=10367436&product=11028230>
- Anonymous b, Biobak, Biofarm, referenced 27.4.2008
<http://www.biofarm.fi/content/view/99/62/lang,fi/>
- Anonymous c, Laboratory for Microbial Ecology, Department of Earth, Ecological and Environmental Sciences, University of Toledo, Denaturing Gradient Gel Electrophoresis (DGGE) 12/2004, referenced 9.3.2008. [available in pdf format]
<http://www.eeescience.utoledo.edu/Faculty/Sigler/RESEARCH/Protocols/DGGE/DGGE.pdf>
- Apajalahti J. H. A., Särkilahti L. K., Mäki B. R. E., Heikkinen J. P., Nurminen P. H., Holben W. E., Effective Recovery of Bacterial DNA and Percent-Guanine-Plus-Cytosine-Based Analysis of Community Structure in the Gastrointestinal Tract of Broiler Chickens. Applied and Environmental Microbiology, October 1998, p. 4084-4088
- Axelsson L. Lactic Acid Bacteria; Classification and Physiology. In: Salminen S., von Wright A., Ouwehand A. Lactic Acid Bacteria; Microbiological and Functional Aspects Third Edition, Revised and Expanded CRC Press, 2004 p. 1-67
- Baillon M.L., Marshall-Jones Z.V., Butterwick R.F, Effects of Probiotic *Lactobacillus acidophilus* strain DSM13241 in Healthy Adult Dogs. American Journal of Veterinary Research 65, 2004, p. 338-343
- Beasley S. Isolation, Identification and Exploitation of Lactic Acid Bacteria from Human and Animal Microbiota. Helsinki Yliopistopaino 2004
- Benno Y., Nakao H., Uchida K., Mitsuoka T., Impact of the Advances in Age on the Gastrointestinal Microflora of Beagle Dogs. Journal of Veterinary Medical Science 54, 1992, p. 703-06
- Benyacoub J., Czarnecki G.L., Cavadini C., Sauthier T, Anderson R.E., Schiffrin E.J., von der Weid T., Supplementation of Food with *Enterococcus faecium* (SF68) Stimulated Immune Functions in Young Dogs. Journal of Nutrition 133, 2003, p. 1158-1162
- Brock T.D., Madigan M.T., Martinko J.m., Parker J., Biology of Microorganisms. 7th edition. London: Prentice Hall, 1994
- Brown T., Gene Cloning & DNA Analysis, an Introduction. Fifth Edition. Blackwell Publishing, 2006
Chambers P., Duggan P., Forbes J. M., Heritage J., A rapid, reliable method for the extraction from avian faeces of total bacterial DNA to be used as a template for the detection of antibiotic resistance genes. Journal of Antimicrobial Chemotherapy 47, 2001, p.241-243
- Cariello N.F., Scott J.K., Kat A.G., Thilly W.G., Keohavong P., Resolution of a Missense Mutant in Human Genomic DNA by Denaturing Gradient Gel Electrophoresis and Direct Sequencing Using In Vitro DNA Amplification: HPRT_{Munich}. American Journal of Human Genetics 42, 1988, p. 726-734
- Chambers P., Duggan P., Forbes J. M., Heritage J., A rapid, reliable method for the extraction from avian faeces of total bacterial DNA to be used as a template for the detection of antibiotic resistance genes. Journal of Antimicrobial Chemotherapy (2001) 47, p.241-243

- Chang Y-H., Kim J-K., Kim H-J., Kim W-Y, Kim Y-B, Park Y-H., Selection of a Potential Probiotic Lactobacillus Strain and Subsequent in vivo Studies. *Antonie van Leeuwenhoek*, 80, 2001, p.193-199
- Davis, C.P., Cleven D., Balish E., Yale C.E., Bacterial Association in the Gastrointestinal Tract of Beagle Dogs. *Applied Environmental Microbiology* 34, 1977, p. 194-206
- Endo A., Okada S., Morita H., Molecular profiling of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* species in feces of active racehorses. *Journal of General Applied Microbiology* 53, 2007, p.191-200
- Goldin B.R., Gorbach S.L., The Effect of Milk and Lactobacillus Feeding on Human Intestinal Bacterial Enzyme Activity. *American Journal of Clinical Nutrition* 39, 1984, p. 756-761
- Greetham H.L., Giffard C., Hutson R.A., Collins M.D., Gibson G.R., Bacteriology of the Labrador Dog Gut: a Cultural and Genotypic Approach. *Journal of Applied Microbiology* 93, 2002, p.640-646
- Holzappel W.H., Haberer P., Geisen R., Björkroth J., Schillinger U., Taxonomy and Important Features of Probiotic Microorganisms in Food Nutrition. *American Journal of Clinical Nutrition* 2001, 73, p.365S-373S.
- Liu, S.-Q., Review article: Practical Implications of Lactate and Pyruvate Metabolism by Lactic Acid Bacteria in Food and Beverage Fermentations. *International Journal of Food Microbiology*, 83, 2003, p.115-131
- Mai V., Morris J. G. Jr., Diet Induced Changes in the Colonic Environment and Colorectal Cancer, Colonic Bacterial Flora: Changing Understandings in the Molecular Age. *American Society for Nutritional Sciences* 2003
- Manninen T.J.K., Rinkinen M.L., Beasley S.S., Saris P.E.J., Alteration of the Canine Small-Intestinal Lactic Acid Bacterium Microbiota by Feeding of Potential Probiotics. *Applied and Environmental Microbiology* October, 2006, p.6539-6543
- McCartney A. Application of molecular biological methods for studying probiotics and the gut flora. *British Journal of Nutrition* 88, 2002, Suppl.1, p.s29-s37
- Muyzer G., Smalla K., Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology, mini review. *Antonie van Leeuwenhoek* 73, 1998, p 127-141
- Nübel U., Engelen B., Felske A., Snaidr J., Wieshuber A., Amann R.I., Ludwig W., Backhaus H., Sequence Heterogeneities of Genes Encoding 16S rRNAs in *Paenibacillus polymyxa* Detected by Temperature Gradient Gel Electrophoresis. *The Journal of Bacteriology* 178 , 1996, p. 5636-5643
- Oksanen Jonna, Laktobasillien kartoittaminen koirien ulosteesta sekä tiettyjen kantojen kasvuominaisuuksien testaus. Helsingin ammattikoulu Stadia, 2002
- Ouwehand A.C., Salminen S., Isolauri E., Probiotics: An Overview of Beneficial Effects. *Antonie van Leeuwenhoek* 82, 2002, p.279-289
- Rinkinen M. : The Intestinal Microbiota of Pets: Dogs and Cats. In: Ouwehand A.C.,Vaughan E. (Eds) *Gastrointestinal Microbiology*. Taylor and Francis. New York, NY, USA. 2006, p.371-380

Rinkinen M., Jalava K., Westermarck E., Salminen S., Ouwehand A.C., Interaction Between Probiotic Lactic Acid Bacteria and Canine Enteric Pathogens: a Risk Factor for Intestinal *Enterococcus faecium* Colonization?. *Veterinary Microbiology* 92, 2003, p.111-119

Rinkinen M. Methods for Assessing the Adhesion of Probiotic and Canine Gut-derived Lactic Acid Producing Bacteria to the Canine Intestinal Mucosa *in vitro* and Measuring Mucosal Secretory IgA. Helsinki Yliopistopaino 2004

Saarela M., Mogensen G., Fondén R., Mättö J., Mattila-Sandholm T., Probiotic Bacteria: Safety, Functional and Technological Properties, Review Article. *Journal of Biotechnology* 84, 2000, p. 197-215

Saxelin M., Hapatteet. In: Salkinoja-Salonen M, toim. Mikrobiologian perusteita. Helsingin Yliopiston Soveltavan kemian ja mikrobiologian laitos. Jyväskylä, Gummeruksen kirjapaino, 2002 p.598-610

Sanz Y., Sánchez E., Marzotto M., Calabuig M., Torriani S., Dellaglio F., Differences in faecal bacterial communities in celiac and healthy children as detected by PCR and denaturing gradient gel electrophoresis. *FEMS Immunology and Medical Microbiology* 51, 2007, p. 562-568

Shanahan, F. Probiotics in Inflammatory Bowel Disease-Therapeutic Rationale and Role. *Advanced Drug Delivery Review* 56, 2004, p.809-818

Suominen I., Ollikka P., Yhdistelmä-DNA-tekniikan perusteet. Opetushallitus, 2003

Vaughan E., de Vries M., Zoetendal E., Ben-Amor K., Akkermans A., de Vos W., The Intestinal LABs. *Antonie van Leeuwenhoek* 82, 2002, p. 341-352

Walter J., Tannock G., Tilsala-Timisjarvi A., Rodtong S., Loach D., Munro K., Alatossava T., Detection and Identification of Gastrointestinal *Lactobacillus* Species Using Denaturing Gradient Gel Electrophoresis and Species-Specific PCR Primers. *Applied and Environmental Microbiology*, Jan 2000, p. 297-303

Wang H-F., Zhu W-Y., Yao W., Liu J-X., DGGE and 16S rDNA sequencing analysis of bacterial communities in colon content and feces of pigs fed whole crop rice. *Anaerobe* 13, 2007, p. 127-133

Zoetendal E.G., Collier C.T., Koike S., Mackie R.I., Gaskins H.R., Diet Induced Changes in the Colonic Environment and Colorectal Cancer, Molecular Ecological Analysis of the Gastrointestinal Microbiota. A Review. *American Society for Nutritional Sciences* 2004

Zoetendal E.G., von Wright A., Vilpponen-Salmela T., Ben-Amor K., Akkermans A.D., de Vos W.M., Mucosa-associated Bacteria in the Human Gastrointestinal Tract Are Uniformly Distributed Along the Colon and Differ from the Community Recovered from the Faeces. *Applied and Environmental Microbiology* 68, 2002, p. 3401-3407

Acknowledgements

My deepest gratitude is due to my supervisor, Adjunct Professor Minna Rininen, who gave me the opportunity to become part of this project. My sincere thanks go to my second supervisor, Dr. Shea Beasley for her advice. I also thank Titta Manninen for her advice and assistance with laboratory work.

My warm gratitude is owed to Dr. Tellervo Korhonen for her advice.

Special warm thanks are owed to my mother Tuula Peiponen.

Last, but not least, my heartfelt thanks go to Antti for being there.