Exploring the microbiota of the gastrointestinal tract

Xu, Jie

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Exploring the microbiota of the gastrointestinal tract

Jie Xu

Food Hygiene
Division of Applied Nutrition and Food Chemistry
Department of Food Technology, Engineering and Nutrition
Faculty of Engineering, Lund University
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Faculty opponent
Senior research fellow Dr Karen Scott
Rowett Institute of Nutrition and Health, University of Aberdeen
United Kingdom

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Abstract: Balanced microbiota of the gastrointestinal (GI) tract is important for maintaining health of the host. Altered gut microbiota have been found to be associated with various life-style induced and other intestinal inflammatory diseases. Gut microbiota is viewed as a metabolic organ and considered as new target for therapies. This thesis describes the work on exploring the microbiota of the GI tract under different conditions. Under the functional food concept, probiotics, prebiotics, food component with prebiotic potientials were used as means to modulate the microbiota of the GI tract. Murine models are commonly used to study the role of the microbiota. However, difference in genetic backgrounds, husbandry conditions may affect the microbiota composition and contribute to the different outcomes of a dietary intervention. We observed that two substrains of C57BL/6 mice fed the same diet harbored different microbiota. In agreement with other studies, this indicates that not only diet but other environmental factors are involved in shaping the gut microbiota. We have investigated the impact of high fat diet on the microbiota and tested multiple functional foods with the aim of improving the host health conditions. We found that high fat diet reshaped the gut microbiota using a mouse model. For example, *Allobaculum*-like bacteria was decreasing whereas *Akkermansia*-like bacteria were increasing with high fat feeding in C57BL/6 mice. Supplementations of green tea and *Lactobacillus plantarum* HEAL19 attenuated high fat-induced inflammation and altered the gut microbiota composition. Combining the *L. plantarum* HEAL19 with different doses of green tea resulted in a gradient shift in the microbiota and different impact on certain bacterial species. Addition of the dietary fibers i.e. pectin or guar gum shifted the gut microbiota differently. Pectin fed rats had significant reduction in weight gain and had increased abundance of *Lachnospiraceae* and an unidentified bacterial group. Berries alone or with probiotics were tested under different host health conditions including healthy, hypertensive and inflammatory state. In murine models, raspberry increased bacterial diversity in the microbiota of rats when compared to blackcurrant. The addition of the *L. plantarum* HEAL19 to the berries did not induce profound effect on the gut microbiota. Fermented bilberries by the *L. plantraum* HEAL19 showed blood pressure lowering effect in healthy and L-NAME-induced hypertensive rats and altered the gut microbiota composition. Supplementation of bilberries protected against inflammation and oxidative stress in an ischemia–reperfusion-mice model and altered cecal microbiota. The addition of probiotics did not have a profound effect on either the health improvement or gut microbiota composition. Three-month intake of dietary supplements containing either *L. plantarum* HEAL19 or *L. plantarum* HEAL19 plus fermented bilberries did not have an obvious impact on the oral and fecal microbiota of hypertensive research persons. Both the oral and the fecal microbiota were relatively stable and the two most fluctuating bacterial taxa in the fecal microbiota were *Bacteroides* and unclassified *Rikenellaceae*. In a dextran sodium sulfate (DSS)-induced colitis mouse model, colonic mucosa associated microbiota were different from the healthy controls. Total load of bacteria and the amount of *Akkermansia* and *Desulfovibrio* were significantly higher in the mice with colitis than the healty controls. In contrast *Lactobacillus* was significantly reduced in colitis group. Moreover, the prevelence of *Enterobacteraiceae* was significantly higher in the colitis group. Ileal pouch microbiota of former patients with ulcerative colitis one year after surgery was colonized predominantly with *Fimicutes* at phylum level. The most abundant genera during the first year after surgery were found to be *Clostridium, Blautia, Roseburia, Lachnospira,* unclassified *Lachnospiraceae*, *Bacteroides, Faecalibacterium,* unclassified *Petostreptococcaceae* and *Megamonas*. In conclusion, diet and other environmental factors are involved in shaping the microbiota of the GI tract. Different diet components have different influence on the microbiota composition. In general, the intestinal *Bacteroidales* taxa were the most active responders for the diet treatments.

Key words: Microbiota, Probiotics, Blood pressure, High fat diet, Oat, Green tea, Berry, Fiber, Exopolysaccharides, Ulcerative colitis

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Division of Applied Nutrition and Food Chemistry
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Contents

Abstract

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Under the functional food concept, probiotics, prebiotics, food component with prebiotic potientials were used as means to modulate the microbiota of the GI tract. Murine models are commonly used to study the role of the microbiota. However, difference in genetic backgrounds, husbandry conditions may affect the microbiota composition and contribute to the different outcomes of a dietary intervention. We observed that two substrains of C57BL/6 mice fed the same diet harbored different microbiota. In agreement with other studies, this indicates that not only diet but other environmental factors are involved in shaping the gut microbiota.

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Berries alone or with probiotics were tested under different host health conditions including healthy, hypertensive and inflammatory state. In murine models, raspberry increased bacterial diversity in the microbiota of rats when compared to blackcurrant. The addition of the *L. plantarum* HEAL19 to the berries did not exert profound effect on the gut microbiota. Fermented bilberries by the *L. plantraum* HEAL19 showed a blood pressure lowering effect in healthy and L-NAME-induced

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Three-month intake of dietary supplements containing either *L. plantarum* HEAL19 or *L. plantarum* HEAL19 plus fermented bilberries did not have an obvious impact on the oral and fecal microbiota of hypertensive research persons. Both the oral and the fecal microbiota were relatively stable and the two most fluctuating bacterial taxa in the fecal microbiota were *Bacteroides* and unclassified *Rikenellaceae*.

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Ileal pouch microbiota of former patients with ulcerative colitis one year after surgery was colonized predominantly with *Fimicutes* at phylum level. The most abundant genera during the first year after surgery were found to be *Clostridium, Blautia, Roseburia, Lachnospira,* unclassified *Lachnospiraceae*, *Bacteroides, Faecalibacterium,* unclassified *Petostreptococcaceae* and *Megamonas*.

In conclusion, diet and other environmental factors are involved in shaping the microbiota of the GI tract. Different diet components have different influence on the microbiota composition. In general, the intestinal *Bacteroidales* taxa were the most active responders for the diet treatments.

Popular Science Abstract

Like earth is home for us, our gastrointestinal (GI) tract is home for microbes. All the bacteria that are living there are called "microbiota" and the total DNA they contain is called "microbiome". The GI microbiota outnumbers human cells by factor of ten and the microbiome is at least 100 times larger than our human genome. A wide range of metabolic activities are performed by our gut microbiota, such as aid digestion by degrading dietary fibers into smaller nutrients which can be absorbed by our intestinal cells, synthesize vitamins, and educating our immune system. Thus, a balanced GI microbiota is important for maintaining health. An extensive research have shown that an altered GI microbiota is associated with obesity, type 2 diabetes, inflammatory bowel diseases and other illnesses. This thesis describes the work on exploring the GI microbiota under different conditions.

We studied the response of the microbiota to high fat diet and inflammation. In order to guide the microbiota to promote health, we used multiple food or food components such as probiotic bacteria (beneficial bacteria, such as those in yogurt), fibers, oat, green tea leaves and berries. Much of the work has been done on animal models.

When we fed oat containing diet to two types of genetically related mice, only one type of mice showed lowered cholesterol. At the same time these mice had a different microbiota composition. It showed that not only the diet but also other environmental factors are involved in shaping the gut microbiota. It also indicates that the microbiota composition may contribute to the different outcomes of a dietary intervention.

High fat diet changed gut microbiota composition dramatically in mice. Supplementations of green tea and probiotic bacteria (a strain of *Lactobacillus plantarum*) to a high fat diet re-shaped the microbiota and attenuated inflammation. A higher diversity is often found in a balanced and healthy ecosystem. We found that probiotic bacteria together with lower amount of green tea increased the microbiota diversity (i.e. the number of different species of bacteria) to a high degree; whilst with higher amount of green tea the Gram negative (bacteria with a certain cell wall structure) of the family *Enterobacteriaceae* (comprising many harmful bacteria) was reduced.

Addition of dietary fibers such as pectin or guar gum induced different responses of the gut microbiota. Interestingly, pectin fed rats had a significant reduction in weight gain and had increased number of certain bacteria including those that can degrade pectin.

Different berries had different influences on the gut microbiota as well. Raspberry increased diversity of the microbiota when compared to blackcurrant, while addition

of the probiotic strain *L. plantarum* HEAL19 to the berries did not show any profound effect. Bilberries (European blueberry) fermented by lactobacilli showed blood pressure lowering effect in healthy and hypertensive rats and altered the gut microbiota compostion. Moreover, bilberries showed protective effect against inflammation and oxidative stress and altered the gut microbiota in mice. The addition of probiotic bacteria did not result in a profound effect on either the health improvement or gut microbiota compostion. Three-month intake of dietary supplements containing probiotic bacteria or bilberries fermented with the same bacteria did not have an obvious impact on the oral and fecal microbiota of individuals with high blood pressure. Both the oral and the fecal microbiota were relatively stable, while two groups of bacteria in the feces varied in numbers by time. Those bacteria were *Bacteroides* and members belonging to unclassified *Rikenellaceae*.

In a mice model mimicking human ulcerative colitis, the microbiota associated to the colonic mucosa were different from that of healthy ones. The total load of bacteria, the amount of the two bacterial genera *Akkermansia* and *Desulfovibrio* and the prevelance of *Enterobacteraiceae* (all of these three are Gram negative bacteria and might be considered as less healthy ones) were significantly higher in the mice with inflammation in colon than in the healthy ones. In contrast, health promoting *Lactobacillus* was significantly reduced in group with inflammation in colon. The mucosa of ileal pouch (that is a pouch that the surgeon makes from a part of the small intestine to replace a colon that has been removed) from former patients with ulcerative colitis one year after surgery was colonized predominantly with a division of bacteria called *Fimicutes* but also bacteria that have been seen in connection to ulcerative colitis were present in some patients.

In conclusion, diet and other environmental factors are involved in shaping the microbiota of the GI tract. Different diet components have different influences on the microbiota composition, which affects our health. Dietary interventions may exert health promoting effects by guiding the microbiota to develop towards a more balanced and healthy ecosystem.

List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. **Xu, J.**, Ahren, I. L., Prykhodko, O., Olsson, C., Ahrne, S., & Molin, G. (2013). Intake of Blueberry Fermented by *Lactobacillus plantarum* Affects the Gut Microbiota of L-NAME Treated Rats. *Evid Based Complement Alternat Med, 2013*, 809128. doi: 10.1155/2013/809128

II. LazouAhrén, I., **Xu, J.**, Önning, G., Olsson, C., Ahrné, S., & Molin, G. (2013). *Antihypertensive activity of blueberries fermented by Lactobacillus plantarum DSM 15313 and effects on gut microbiota in healthy rats.* (Submitted)

III. **Xu, J.**, LazouAhrén, I., Olsson, C., Jeppson, B., Ahrné, S., & Molin, G. (2013). *Oral and fecal microbiota in volunteers with hypertension in a placebo controlled trial with probiotics and fermented bilberries.* (Submitted)

IV. **Xu, J.**, Falk, A., Olsson, C., Ahrné, S., Thorlacius, H., Jeppsson, B., & Molin, G. (2013). *Ileal pouch microbiota after surgery of former ulcerative colitis patients.* (Manuscript)

Nine other studies where the author has performed the microbiota analyses are referred and discussed in the thesis. These supplementary studies are referred to as Study A to Study I.

Study A. Olsson, C., **Xu, J.**, Andersson, U., Ahrné, S., & Molin, G. (2013). *A high fat diet promotes the growth of Akkermansia and Bilophila in C57BL6 mice.* (Manuscript)

Study B. Andersson, K. E., Axling, U., **Xu, J.**, Sward, K., Ahrne, S., Molin, G., Holm, C., & Hellstrand, P. (2013). Diverse effects of oats on cholesterol metabolism in C57BL/6 mice correlate with expression of hepatic bile acid-producing enzymes. *Eur J Nutr, 52*(7), 1755-1769. doi: 10.1007/s00394-012-0479-1

Study C. Lindström, C., **Xu, J.**, Oste, R., Holst, O., & Molin, G. (2013). Oral administration of live exopolysaccharide-producing Pediococcus parvulus,

but not purified exopolysaccharide, suppressed *Enterobacteriaceae* without affecting bacterial diversity in ceca of mice. *Appl Environ Microbiol, 79*(16), 5030-5037. doi: 10.1128/aem.01456-13

Study D. Jakobsdottir, G., **Xu, J.**, Molin, G., Ahrne, S., & Nyman, M. (2013). High-Fat Diet Reduces the Formation of Butyrate, but Increases Succinate, Inflammation, Liver Fat and Cholesterol in Rats, while Dietary Fibre Counteracts These Effects. *PLoS One, 8*(11), e80476. doi: 10.1371/journal.pone.0080476

Study E. Axling, U., Olsson, C., **Xu, J.**, Fernandez, C., Larsson, S., Strom, K., Ahrne, S., Holm, C., Molin, G., & Berger, K. (2012). Green tea powder and *Lactobacillus plantarum* affect gut microbiota, lipid metabolism and inflammation in high-fat fed C57BL/6J mice. *Nutr Metab (Lond), 9*(1), 105. doi: 10.1186/1743-7075-9-105

Study F. **Xu, J.**, Olsson, C., Axling, U., Berger, K., Holm, C., Ahrné, S., & Molin, G. (2013). Green tea powder and *Lactobacillus plantarum* in the diet increase diversity and affect composition of the gut microbiota in mice. (Manuscript)

Study G. Jakobsdottir, G., Blanco, N., **Xu, J.**, Ahrne, S., Molin, G., Sterner, O., & Nyman, M. (2013). Formation of short-chain Fatty acids, excretion of anthocyanins, and microbial diversity in rats fed blackcurrants, blackberries, and raspberries. *J Nutr Metab, 2013*, 202534. doi: 10.1155/2013/202534

Study H. Jakesevic, M., **Xu, J.**, Aaby, K., Jeppsson, B., Ahrne, S., & Molin, G. (2013). Effects of bilberry (*Vaccinium myrtillus*) in combination with lactic acid bacteria on intestinal oxidative stress induced by ischemia-reperfusion in mouse. *J Agric Food Chem, 61*(14), 3468-3478. doi: 10.1021/jf400203h

Study I. Håkansson, Å., Baridi, A., Tormo-Badia, N., **Xu, J.**, Molin, G., Hagslätt, M.-L., Karlsson, C., Jeppsson, B., Cilio, C., & Ahrné, S. (2013). Immunological alteration and changes of gut microbiota after dextran sulphate sodium (DSS) administration in mice. (Manuscript)

Note: Overview of the studies including the types of samples, background of diet or treatment and analysis methods are summarized in **Table 1** (page 30).

The author's contributions

Paper I. The author, Jie Xu, designed and performed the analysis of the intestinal microbiota, analyzed the data, and was responsible for evaluating the results and writing the manuscript.

Paper II. The author, Jie Xu, designed and performed the analysis of the intestinal microbiota, participated in evaluating the data and wrote the manuscript in collaboration with Irini Lazou Ahrén.

Paper III. The author, Jie Xu, designed and performed the analysis of the oral and fecal microbiota, analyzed the data, and was responsible for evaluating the results and writing the manuscript.

Paper IV. The author, Jie Xu, took part in planning the study, designed and performed the analysis of the ileal pouch mucosal microbiota, analyzed the data, and was responsible for evaluating the results and writing the manuscript.

Abbreviations

ALAT	Alanine aminotransferase
ATCC	American Type Culture Collection
BMI	Body mass index
bp	base pair
BP	Blood pressure
CCUG	Culture Collection, University of Gothenburg, Sweden
CFU	Colony-forming unit
DBP	Diastolic blood pressure
DGGE	Denaturing Gradient Gel Electrophoresis
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DSS	dextran sodium sulfate
GI	Gastrointestinal
HEAL19	Lactobacillus plantarum HEAL19
HFD	High fat diet
LFD	Low fat diet
L-NAME	N^G-nitro-L-arginine methyl ester
LPS	Lipopolysaccharides
nt	nucleotide
OPLS	Orthogonal partial least squares projections to latent structures
PCA	Principal component analysis
PLS	Partial least squares projections to latent structures
qPCR	quantitative polymerase chain reaction
RDP	Ribosomal Database Project
SBP	Systolic blood pressure
SCFA	Short-chain fatty acids
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TLR	Toll-like receptor
T-RFs	Terminal Restriction Fragments
UC	Ulcerative colitis

Introduction

Microbiota of GI tract in healthy humans

A diverse microbiota resides along the human gastrointestinal (GI) tract. The acquisition of the intestinal microbiota is thought to begin at birth, some evidence suggest maybe even before birth [1]. The first colonizers are often more oxygen tolerant bacterial species belonging to *Staphylococcus, Streptococcocus, Enterobacteriaceae, Enterococcus, Bifidobacterium, Lactobacillus* etc. A shift towards more anaerobic bacteria such as *Bacteroides* and *Clostridia* occurs later in the first year of life [1-4]. However, the inter-individual difference is large between infants but the stability of the individual microbiota is maintained within the baby and by the age of one, the composition starts to resemble an adult microbiota [2]. The colonization of the bacterial species are influenced by many factors such as delivery mode (cesarean vs. vaginal delivery), breast feeding or formula feeding, genetic factors, environmental exposures and antibiotic treatment [2, 5-8]. By aging, the microbiota continues to mature and forms a highly complex and diverse ecosystem. The dominant bacterial taxa present in adult microbiota at phylum level are *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria* and *Verrumicrobia* at lesser amount. At lower taxonomic level, bacterial species belonging to genera *Bacteroides, Eubacterium, Clostridum* and *Ruminococcus, Faecalibacterium, Dorea, Alistipes* are commonly found abundant in adult microbiota with most of the species uncultivable [9, 10]. In an adult, bacterial cells outnumber human cells by factor of 10 and the size of microbiome is estimated at least 100 times larger than human genome [11]. Thus, the gut microbiota can be viewed as microbial organ which possesses the metabolic functions that we do not have by our own account [11, 12].

The human GI tract provides multiple habitats for bacteria starting from the oral cavity down to the large intestine. Segata et al. (2012) studied the microbiota of different sites along the GI tract and found four distinct types of microbiota. Oral microbiota could be divided into three groups depending on the sampling sites. For instance, buccal mucosa was predominantly occupied by *Streptococcus* whereas saliva and sub and supra-gingival plaque associated microbiota had more even distribution of the different genera *Streptococcus, Veillonella, Prevotella, Neisseria, Fusobacterium, Actinomyces, Leptotrichia, Corynebacterium, Capnocytophaga, Rothia* and

Porphyromonas. The last four genera were found abundant in sub and supra-gingival plaque. The other distinct group was the gut microbiota analyzed from fecal samples, where *Bacteroides* was the predominant genus [13]. Similarly, various bacterial diversity levels have been observed at different sites of oral cavity [14].

In comparison to the oral microbiota, esophagus harbors a less diverse microbiota with most of the species cultivable. Comparable with the oral mucosa associated bacteria, *Streptococcus* was the most abundant member in healthy individuals [15-17].

The harsh environment of the stomach challenges bacterial survival and colonization. However, certain bacteria are adapted to the harsh conditions and form a distinct stomach microbiota. With the development of sequencing technique, a more diverse microbiota than expected has been found in the stomach. The dominant phyla were *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Fusobacteria* and sometimes presence of taxa "TM7", *Deferribacteres, Deinococcus-Thermus, Chlamydia* and *Cyanobacteria* have been reported [18-20]. However, in the presence of *Helicobacter pylori*, a well studied pathogen that can cause various gastric diseases [21], could become totally dominant and thus dramatically reduce the bacterial diversity [18, 19]. When *H. pylori* was absent the genera *Streptococcus, Prevotella, Actinomyce, Gemella Propionibacterium and Lactobacillus* have been found abundant in some studies [18-20].

Bacteria residing in small intestine increase in density and diversity along the three sections, duodenum (10^1-10^3 CFU/ml), jejunum and ileum (10^4-10^7 CFU/ml) [12]. Although the gastric acid is largely neutralized in duodenum, the luminal pH is still acidic (pH 4-5) and the influx of bile and pancreatic juice hinder the colonization of the bacteria. Thus, acid and bile tolerant bacterial species from *Veillonella*, *Lactobacillus*, and *Clostridium* were found in duodenum [15]. In jejunum and upper ileum, bacterial species belonging to *Streptococcus, Lactobacillus, Enterobacter, Bacteroides, Fusobacterium, Proteus,* and *Staphylococcus* were detected in different studies [15, 22, 23]. Distal ileal microbiota was more similar to that of colon, where proportion of *Clostridium* XIVa group and *Bacteroidetes* increased [23, 24].

The large intestine is the most densely populated area (10^{11}-10^{12} CFU/ml) [12], and again the bacterial load in lumen increases from cecum to distal colon. When cecal and fecal microbiota from healthy people was compared, there was a 100 fold difference especially with the lower number of strict anaerobes in cecum [25]. Even though fecal microbiota cannot completely reflect the microbiota structure in the intestinal lumen, with the advantage of non-invasive and simple sampling, fecal samples have commonly been used to study the large intestinal microbiota in healthy humans. At high taxonomic level, phyla *Firmicutes* and *Bacteroidetes* were the most abundant bacteria of the fecal microbiota. Arumugam et al. (2011) reported that the

human gut microbiota could be clustered into three enterotypes depending on the abundant level of *Bacteroides, Prevotella* or *Ruminococcus* regardless of nationality, gender, age or body mass index (BMI) [26]. One factor for the enterotype clustering, suggested by another study, was the long time diet pattern. It was reported that high animal protein and fat intake was linked to the *Bacteroides* enterotype whereas carbohydrate rich food intake was linked to the *Prevotella* enterotype [27]. However, other studies failed to identify the clear clustering of enterotypes, but instead observed an abundance gradient of the dominant genera [28, 29].

In general, a high level of variation in microbiota exists among individuals since early life, at the same time, the relative stability of the microbiota is maintained within an individual through time.

Gut microbiota and metabolic syndrome

Metabolic syndrome (MetS) defined by the International Diabetes Federation (IDF) consists of central obesity (or BMI>30 kg/m^2) together with two of any of the conditions: raised triglycerides (> 150 mg/dL or 1.7 mmol/L) or specific treatment for this lipid abnormality; reduced HDL-cholesterol (<40 mg/dL or 1.03 mmol/L in men; <50 mg/dL or 1.29 mmol/L in women) or specific treatment for this lipid abnormality, raised blood pressure (SBP≥130, DBP≥85) or treatment of previously diagnosed hypertension; raised fasting plasma glucose (≥100 mg/dL or 5.6 mmol/L) or previously diagnosed type 2 diabetes [30]. Metabolic syndrome is a risk factor for type 2 diabetes and cardiovascular diseases, which are the leading causes of deaths over the world.

Growing evidence points to that gut microbiota may play a role in the development of MetS. In 2004, Bäckhed et al. firstly showed that microbiota could affect the fat storage of the host. They found that conventional C57BL/6J mice, although consumed less feed, had 42% more body fat than the counterpart germ free mice. When the germ free mice were conventionalized with the cecal microbiota of the conventional mice, significant increase in their body fat content was observed [31]. Later, the same group found that conventionalized germ free mice gained significantly more weight than germ free counterpart when fed western diet (high fat, high sugar). They stated that germ free animals were protected from diet-induce obesity. One of the possible mechanisms could be the higher expression of Fiaf, a lipoprotein lipase (LPL, hydrolyzes triglycerides into free fatty acids) inhibitor in germ free mice [32]. The result of increased body fat in conventional mice compared to germ free mice fed with a western diet could be reproduced by another group [33]. However, in the same study, when fed high fat diet, germ-free mice gained similar or more weight than

conventional mice at two experimental settings. High fat diet fed mice had significantly higher body fat content than those fed with western diet. Even though the energy percent of the macronutrient were similar in the high fat and western diets, the types of carbohydrate and lipids differed between the two diets. For example, western diet contained *trans*-fatty acids and more than three times of sucrose when compared to the high fat diet (no *trans*-fatty acids). The authors also found a higher expression of intestinal Fiaf at mRNA level in germ free mice than conventional mice. However, they did not detect difference at the protein level. The authors concluded that absence of intestinal microbiota does not protect from diet-induce obesity [33].

A shift in microbiota composition associated with obesity was seen as an increase in the abundance of *Firmicutes* at the expensive of *Bacteroidetes* in genetically obese mice in comparison to the lean mice fed the same diet, and also in humans with calorie-restricted diets where weight loss was positively correlated with increase of *Bacteroidetes* [34, 35]. Again the same research group, this time colonized the germ free mice with human fecal microbiota and fed mice either low fat, plant polysaccharide rich diet or high fat, high sugar western diet. The decreased ratio of *Bacteroidetes/Firmicutes* was observed in the western diet fed mice and the significantly increased bacteria were from class *Erysipelotrichi* belonging to *Fimicutes* [36]. Similar findings were observed in conventional mice fed high fat diet or western diet where the increase of *Erysipelotrichaceae* was the main driving force of the increase in *Firmicutes* [33]. In another study, *Erysipelotrichaceae* (phylum *Firmicutes*) was found mostly in obese individuals compared to normal weight and post-gastric bypass individuals. However, *Bacteroidetes* had somewhat higher abundance in obese individuals than in normal weight individuals [37]. Another study also questioned the link between the increased ratio of *Firmicutes/Bacteroidetes* and obesity by estimating *Bacteroidetes* load in feces from obese and non-obese individuals with fluorescent in situ hybridization (FISH) method. No difference was found in the proportion of *Bacteroidetes* between obese and non-obese individuals and no correlation was found between weight loss and change in *Bacteroidetes* [38]. Schwiertz et al., studied SCFA profile in lean, overweight and obese individuals and quantified several fecal bacterial groups. They found that the total amount of SCFA and propionate was higher in overweight and obese group than lean group. They also stated that the proportion of *Firmicutes/Bacteroidetes* was decreasing in the overweight and obese groups [39]. Schwiertz et al., used qPCR to quantify the typical genus or group belonging to the two phyla and based on those they calculated the ratio of the *Fimicutes/Bacteroidetes*. However, this mode of procedure may lead to a biased result by underestimating the proportion of the bacteria that are not included in qPCR enumeration.

Cani et al. were first to find that an altered gut microbiota may lead to low grade inflammation and that in turn could trigger the development of metabolic diseases [40-42]. It was found that high fat diet (72% energy from fat) feeding induced elevated plasma lipopolysaccharide (LPS) level in mice, a condition defined as

"metabolic endotoxemia" [40]. High fat diet also induced elevated endotoxins in humans [43]. LPS is an outer membrane component of gram negative bacteria, and evokes immune activation in mammalian host. LPS is generally composed of 4 parts: *O*-polysaccharide chain, outer core, inner core and lipid A in the order of outermost part to the surface of the bacteria. The *O*-polysaccharide chain is a major antigen targeted by host immune cells and the lipid A is responsible for the toxicity. LPS activates toll-like receptor 4 (TLR4) and sometimes TLR2 signaling pathway in the immune cells. The structures and endotoxicity of LPS molecules vary among different bacterial species and strains [44]. In the high fat diet fed mice, the metabolic endotoxemia was accompanied by reduced level of certain gram negative ("*Bacteroides-like*" bacteria that are closely related to the *Bacteroides-Prevotella* group) and gram positive bacteria (i.e. "*Eubacterium rectale-Clostridium coccoides* group" and bifidobacteria) [40]. Normalization of the endotoxemia was achieved when oligofructose was supplemented to the high fat diet which boosted the growth of intestinal bifidobacteria [41]. In a later study, by the same group, involvement of gut microbiota in controlling of the intestinal permeability was discovered [42]. Moreover, the gut microbiota has been shown to modulate insulin resistance in toll-like receptor 2 (TLR2) deficient mice [45]. In this study, a series of experiments were carried out to investigate the effect of gut microbiota on the metabolic parameters and the underlying mechanism. Regardless of diets (standard chow or high fat diet), TLR2 knockout (KO) mice developed features that resembles metabolic syndrome and had altered microbiota characterized by a 3-fold increase in *Fimicutes* and slight increase in *Bacteroidetes* compared to wild type (WT) control. The change in gut microbiota was accompanied by increase in LPS absorption, inflammatory parameters, insulin resistance and obesity. These outcomes could be reproduced in WT mice by transplantation of the cecal microbiota of TLR2 KO mice [45]. It was also reported in humans that transplantation of fecal microbiota from lean donors to individuals with metabolic syndrome resulted in increased insulin sensitivity [46].

In patients with type 2 diabetes, bacterial diversity calculation based on gradient gel electrophoresis (DGGE) was not significantly different from healthy individuals. However, intra-group similarity was higher than the inter-group similarity which indicates the compositional difference in the microbiota between diabetic and healthy individuals. Subsequent sequencing of the DGGE bands showed that at genus level, *Bacteroides* was most abundant in diabetic patients whereas *Prevotella* was most abundant in healthy persons. Additionally, *Parabacteroides* was only detected in the diabetic group. A significant reduce in the amount of *Bifidobacterium* measured by qPCR was also observed in the diabetic group [47]. In contrast, *Bacteroides* was found half as abundant in the diabetic group when compared to normal glucose group and pre-diabetic group according to the result from 16S rDNA amplicon pyrosequencing. PCA analysis showed no clear separation between the diabetic and the other two groups and no significant difference in diversity was found between the groups. The

abundance of *Akkermansia muciniphila*, and *Faecalibacterium prausnitzii* were found higher in the normal glucose group [48]. One recent study also reported similar findings regarding to *Akkermanisa muciniphila* in mice [49]. Difference in the gut microbiota was also observed between diabetic and non-diabetic persons within a wide range of BMI (23~48). The proportion of phylum *Firmicutes* and class *Clostridia* were significantly reduced in the diabetic group and the plasma glucose level was positively correlated with the ratio of *Bacteroidetes/Fimicutes* and *Betaproteobacteria*, which was enriched in the diabetic group [50].

The search for the role of the microbiota in the development and regulation of metabolic syndrome is growing. The outcomes of the research so far clearly shows that the diet has profound effect on shaping gut microbiota, however the interaction between gut microbiota and host physiology is complicated which is indicated by the conflicting results in literature. Further studies and more efforts are needed to clarify the role of the microbiota and understand the underlying mechanism.

Gut microbiota and ulcerative colitis

Ulcerative colitis (UC) is a chronic relapsing intestinal inflammatory disease of unknown cause that specifically affects colonic and rectal mucosa. Together with Crohn's disease, which can affect anywhere in the GI tract, the two are categorized as inflammatory bowel disease (IBD).The involvement of the gut microbiota in the pathogenesis of UC is suggested in many studies.

Much effort has been made to characterize the dysbiosis (imbalanced gut microbiota) in the UC patients and to identify specific bacterial groups that are involved in the pathogenesis. In UC patients, "*Clostridium coccoides* group" in feces was significantly reduced compared to healthy individuals [51]. *Campylobacter* were found predominant in the colonic mucosa compared to healthy controls. Especially two species, *Campylobacter concisus* and *Campylobacter ureolyticus*, were suspected for the involvement of triggering an inflammatory cascade leading to UC [52]. Sequence analysis of 16S rRNA gene which was amplified from colonic mucosa samples taken from 12-year old young UC patient showed that most sequences were assigned to *Gamma-proteobacteria*, followed by *Clostridium* clusters IV and XIVa. High incidence of *Enterobacteriaceae*, *Bacteroides fragilis*, and *Faecalibacterium prausnitzii*-like bacteria were detected [53]. In another study, T-RFLP analysis of fecal samples from UC patients and healthy individuals resulted in separate clusters and difference also existed between active and remission UC patients. Active UC patients were predominated by *Eubacterium* and *Fusobacterium* whereas remission UC patients were dominated by *Lactobacillus* [54]. However, in that study the T-RFs were identified by

computer simulation, thus it may yield biased identification. In another study, also using T-RFLP, a significant less diverse rectal mucosa-associated microbiota was observed in active UC patients compared to inactive UC patients, but no specific T-RF was associated with active UC patients [55]. Significantly decreased fecal microbiota diversity (based on DGGE band pattern) was also reported in UC patients when compared to healthy individuals [56]. Unstable fecal microbiota in UC patients with clinical remission was observed in a one-year follow up study. The fecal microbiota was analyzed with DGGE and the result showed that bacterial diversity was lower in patients than healthy controls and similarity index kept dropping in patients by time whereas healthy individuals maintained the stable microbiota [57]. Fluctuation of bacterial species was found in another longitudinal study, where rectal mucosa associated microbiota of active UC patients was followed up over one year. High clinical activity indices (CAI) and sigmoidoscopy scores (SS) were positively correlated with high densities of mucosa associated *Enterobacteria, Desulfovibrio, Bacteroides* and *Enterococcus faecalis*, whereas negative correlations were found against *Clostridium butyricum, Ruminococcus albus* and *Eubacterium rectale*. The amount of *Lactobacillus* and *Bifidobacterium* were negatively associated with CAI. Moreover, *E. rectale* and *Clostridium clostridoforme* were found to be age dependent. In addition, levels of *F.prausnitzii* continuously increased by time and were only significantly less than non-IBD controls at start of the study and after 3 months. [58]. However, *F.prausnitzii* level increased in UC patients in remission, whereas remained low if the patients relapsed in a 1-year follow up study [59]. *F. prausnitzii* and *Roseburia hominis* (both butyrate-producing) were significantly less abundant in fecal samples taken from UC patients compared to healthy controls. SCFA production was also lower in UC patients. However, no significant correlation was found between the SCFA and the identified bacteria [60].

Fecal microbiota transplantation (FMT) from healthy donor to UC patients did not lead to satisfactory results. Angelberger et al. carried out FMT on 5 UC patients and did 3-month follow up study using 16S rDNA amplicon sequencing. Only one patient showed positive clinical response and harbored a shifted microbiota that resembles donor's marked by enrichment in *F. prausnitzii, Roseburia faecis* and *Bacteroides ovatus.* In other patients, the FMT only resulted in shorter transient changes in similarity between the microbiota of the donors. Furthermore, the dissimilarity increased by time and disease severity was positively correlated with high abundance of *Enterobacteriaceae* and negatively correlated with high abundance of *Lachnospiraceae* [61]. In another FMT study, where 6 patients were involved, temporary improvement two weeks after FMT was achieved regarding to the decrease in stool frequency but FMT failed to achieve clinical remission. In three patients the microbiota shifted towards a structure that is more similar to the donors', however no correlation could be found against clinical response. At higher taxonomic level, a

significant decrease in *Proteobacteria* and increase in *Bacteroidetes* was observed after FMT [62].

Generally, altered gut microbiota with decreased diversity was associated to UC. However, specific causative bacteria have not been identified yet.

Probiotics

Generally, probiotics are live microorganism, when consumed in adequate amount, exert health beneficial effects to host [63]. The idea of ingesting "good" bacteria to improve health dates back to one hundred years ago, when Elie Metchnikoff suggested that consumption of the yogurt fermented by lactobacilli was associated with increased longevity of the host [64]. Probiotics intake has been shown effective on prevention and treatment of gastrointestinal diseases (e.g. various diarrheal diseases) and allergy and atopic diseases of children. Beneficial effects also have been observed on cholesterol lowering and prevention of certain types of cancers [65-67]. Most common probiotics are from the genera *Lactobacillus* and *Bifidobacterium.* In addition, strains from *Lactococcus, Streptococcus* (*Streptococcus thermophilus*), non-pathogenic *Enterococcus*, non-pathogenic *Escherichia coli* and some yeast strains of *Saccharomyces* have also been used as probiotics. The main actions of probiotics reported in literature has been reviewed by Howarth and Wang [68], which includes (1) a shift of microbiota composition towards a more beneficial microbial ecosystem, (2) high adhesion ability to mucosal surfaces and epithelial cells to compete with pathogens, (3) antimicrobial properties against certain pathogens, (4) aid the maintenance of intestinal barrier function, (5) affect cell kinetics such as cell proliferation, apoptosis, and (6) affect immunity [68].

Probiotics and metabolic syndrome

Probiotics have shown promising effects in intervention studies using animal models. Supplementation of *Lactobacillus paracasei* subspecies *paracasei* F19 to high fat diet decreased the body fat in mice [69]. *Lactobacillus reuteri* ATCC PTA 4659 prevented diet-induced obesity in atherosclerosis-susceptible mice [70]. In a rat model, the supplementation of *Lactobacillus plantarum* K68 to high fat-fructose diet was reported to attenuate metabolic syndrome [71]. In another rat model, supplementation of a mix of two probiotic strains, *Lactobacillus curvatus* HY7601 and *L. plantarum* KY1032 to a high-fructose diet, could reverse the risk factors of metabolic syndrome [72]. On the other hand, probiotic intervention trials in humans have not always yielded promising results. Beneficial effects on reducing LDL-cholesterol, decreased

body weight and BMI index were observed in healthy individuals by consuming a yogurt containing multiple probiotic strains for 8 weeks [73]. However, no beneficial effects were seen in a couple of other studies [74-76].

Probiotics and ulcerative colitis

Limited research has been done on probiotic interventions in UC patients. Probiotic *Escherichia coli* Nissle 1917 (EcN) was as effective as anti-inflammatory medicine 5-aminosalicylic acid (5-ASA) in maintaining remission in children and adolescents with UC [77]. Consumption of VSL# (containing 8 strains from *L. casei, L. plantarum, L. acidophilus, L. delbrueckii* subsp. *bulgaricus, B. longum, B. breve, B. infantis* and *Streptococcus salivarius* subsp. *thermophilus*) were able to induce and maintain remission in children with UC [78, 79]. Positive effects of probiotics intervention have been observed in several dextran sodium sulfate (DSS)-induced murine models. *Bifidobacterium infantis* DSM 15158 and *B. infantis* DSM 15159 alone or in combination with oligofructose and inulin [80], *Lactobacillus plantarum* DSM 15313(=HEAL19) or a *L. fermentum* stain (without tannase activity) alone or combined with freeze dried blueberries [81] were able to attenuate the inflammation, and EcN treatment led to a reduced colonic epithelial permeability [82]. In a DSS-induced chronic colitis model, supplementation of blueberry husks combined with a mixture of three probiotic strains (*B. infantis* DSM 15159, *Lactobacillus gasseri* DSM 16737 and *L. plantarum* DSM 15313) to an oat fortified diet attenuated disease activity and the probiotic mixture showed liver protective effect [83].

Functional food

"Functional food" is a concept originated from Japan in 1980s and has also developed in Europe. A food can be regarded as a functional food if the consumption is able to deliver one or more targeted health beneficial functions with the amounts that can normally be expected in a diet [84]. Probiotics, prebiotics or the combination synbiotics could become components of functional foods.

With the aim of achieving synergy effect with probiotic containing food, various substrates are being explored. Dietary fiber rich food, or polyphenol rich food such as green tea and berries with prebiotic potential have sometimes been combined with probiotics in the studies discussed in this thesis.

Dietary fibers are non-starch plant polysaccharides that are resistant to digestion and absorption in the small intestine but fermented by commensal bacteria in the large intestine. Common examples of dietary fibers include cellulose, hemicelluloses, lignin, pectin and beta-glucans. Dietary fibers could be grouped into soluble or insoluble fiber. Soluble fibers such as pectins, gums, inulin-type fructans, are easily fermented by microbiota in larger intestine, whereas insoluble fibers such as lignin, cellulose are fermented to a limited extent. Beneficial effects of dietary fiber intake on obesity and type 2 diabetes have been reviewed by Lattmer and Haub [85].

Green tea and berries are rich sources of polyphenols. Various health beneficial effects have been attributed to the antioxidant properties of the polyphenols [86, 87]. Polyphenols can be classified into phenolic acids, flavonoids, stilbenes and lignans depending on their chemical structures (number of phenol rings and the binding elements between the rings). Flavonoids share a similar chemical structure and can be divided into flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (catechins and proanthocyanidins) [88, 89]. Green tea or whole berries contains other constituents beside polyphenols, i.e. fibers and essential nutrients such as vitamins and minerals.

Depending on the types of the fiber or polyphenols and the amount they are in, they can exert different selection pressure towards the gut microbiota. The interaction between the gut microbiota and its substrate can lead to different physiological consequences.

Aim

The aim of this thesis is to explore the microbiota of the GI tract under different conditions in search for clues about its role in health and disease. The strategy has been to map the microbiota in different studies with similar background, and then to compare and summarize the observations.

Methods

This section provides an overview of the methods (Fig. 1) used in this thesis. For detailed information please see the individual papers.

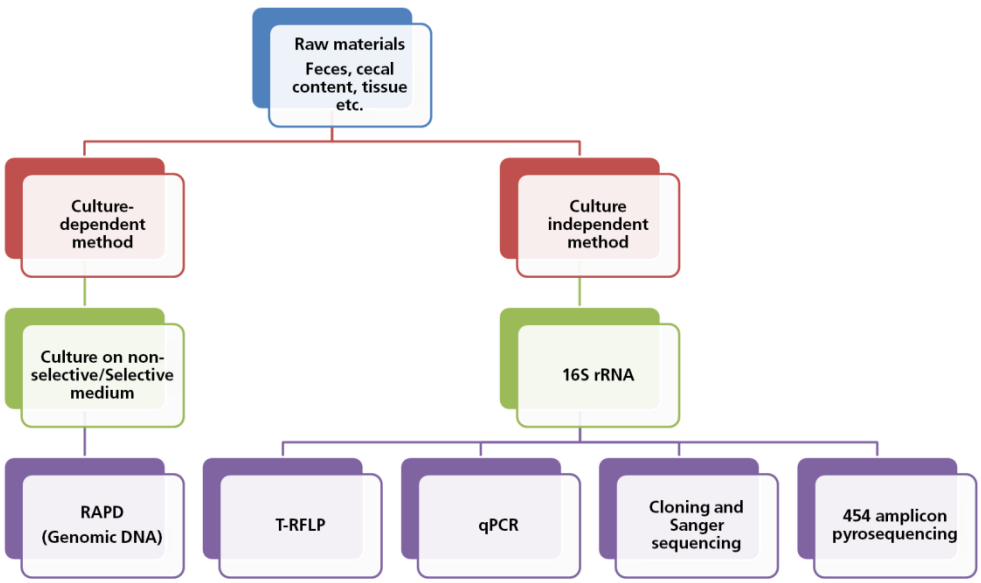

Figure 1. Schematic workflow of the microbiota analyses used in this thesis.

Culture method

Generally cultivation of bacteria from the environmental samples starts with homogenization of the material followed by serial dilution steps. The samples taken from each dilution are plated on non-selective/selective media and incubated at, for example, 37 ºC aerobically or anaerobically. Culturing of *Lactobacillus* and *Enterobacteriaceae* was done in **study E** (Axling et al.). The culture method has advantages for direct enumeration of live bacteria and phenotypic characterization. The disadvantages could be the fact that large proportion of the microbiota along the GI tract is not cultivable, and the culturing can be time consuming and selective media are not completely selective (e.g. Rogosa agar permit growth of non-*Lactobacillus* species [90]). An example of bacterial cultures as colonies on an agar plate is shown in Figure S1.

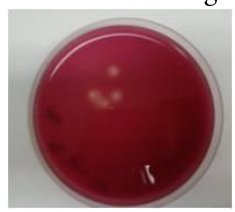

Figure S1. *Desulfovibrio desulfuricans* CCUG 34226 grew on blood agar plate, taken in May 2010

The culture-dependent molecular method

Randomly Amplified Polymorphic DNA

Randomly Amplified Polymorphic DNA (RAPD) is a PCR-based method, unlike the traditional PCR, the target sequence is unknown and a single short arbitrary primer (~10 nt) is used as both forward and reverse primer. If the distance between the primers is too long for PCR to complete or the 3′-ends of the primers are not facing each other, there will not be any PCR product [91](Fig. 2). The PCR reaction starts under low stringency conditions (i.e. annealing at 30 °C) allowing some mismatches. After a few initial cycles, higher annealing temperature can be used to increase the PCR specificity. RAPD was used to identify HEAL19 in **study E**. The advantages of the RAPD method include that there is no need of DNA sequence information and the experiment could be done efficiently with low cost. On the other hand, the disadvantages of RAPD include that it is highly sensitive to experimental conditions (i.e. template DNA, PCR reactions, thermal cycler, etc.) and the RAPD patterns are lab-dependent[92]. An example of the RAPD pattern is shown in Figure S2.

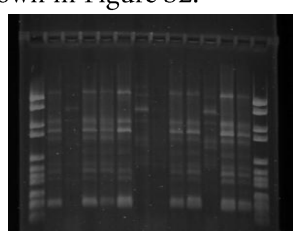

Figure S2. RAPD patterns of lactobacilli isolates, taken in Oct 2008

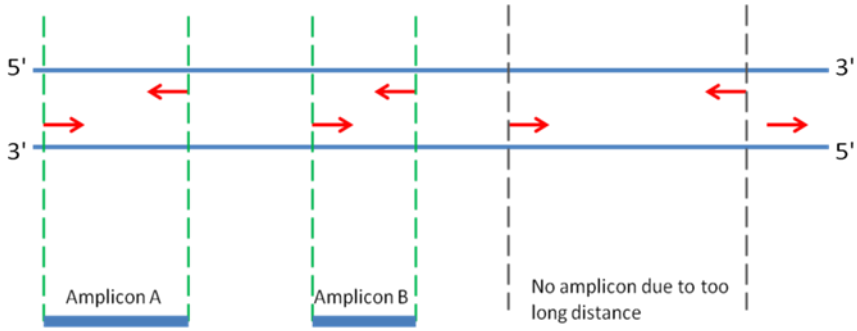

Figure 2. Schematic view of the RAPD-PCR

Culture-independent Methods

DNA extraction

Samples. Bacterial genomic DNA was extracted from pure cultures, feces and cecal content (from mice, rats and humans), intestinal tissues (from small intestine, cecum and colon), mouth swabs, and ileal pouch biopsies in former ulcerative colitis patients. Different isolation methods were used according to the sample type.

From pure cultures. The bacterial cells were pelleted by centrifugation, and followed by washing step with water and were re-pelleted. Then the pellet was resuspended in water, PBS (Oxoid, Basingstoke, UK) or EB buffer (Qiagen, Hilden,Germany). Sterile 2 mm glass beads were added to the suspension and the samples were then shaken for 30-45 minutes in an Eppendorf Mixer (Model 5432, Eppendorf, Hamburg, Germany). Brief centrifugation was done to pellet the cell debris and the supernatant containing bacterial DNA is used for template in PCR.

From other resources. Total DNA was isolated using EZ1 DNA tissue kit (Qiagen) on a BioRobot EZ1 workstation (Qiagen) with EZ1 DNA Tissue Card or on an EZ1 Advanced XL workstation (Qiagen) with EZ1 Advanced DNA Bacteria Card according to the manufacturer's instructions with minor modifications. For example, bead beating step was added to aid the lysis of Gram positive bacteria prior to robot extraction step. For tissues, longer lysis time was applied when it is needed. An excessive amount of fecal or cecal content samples were usually collected. Thus taking materials for DNA analysis from top part of the content or middle part may

introduce bias in comparison of microbial community structure between samples. However, homogenization of feces or cecal content after thawing the frozen material prior to DNA extraction is not practical because efficient handling of sample at low temperature is needed to hinder the overgrowth of microorganisms which may distort the true microbiota structure. To take better homogenized samples, lyophilized fecal samples were used for DNA isolation in paper **III**.

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Procedure. T-RFLP was used to get an overview of the microbiota structure (Fig. 3). Briefly, fluorescently labeled forward primer FAM-ENV1 (5′-FAM-AGA GTT TGA TII TGG CTC AG-3′) corresponding to *E.coli* position 8-27 and a non-labeled reverse primer ENV2 (5′-CGG ITA CCT TGT TAC GAC TT-3′) corresponding to *E.coli* position 1511-1492 was used to amplify the 16S rRNA gene. The amplicon size is ~1504bp spanning V1-V9 region. After purification of the PCR products, four base frequent cutters for example *Msp*I (5′-C⁞CGG-3′) and/or *Alu*I (5′-AG⁞CT-3′) were used in T-RFLP to generate unique patterns with reasonable amount of fragments. Then the digested fragments were analyzed on an ABI 3130xl Genetic analyzer (Applied Biosystems, Foster city, CA, USA) with internal size standard GeneScan LIZ 600 (range 20 – 600 nt, Applied Biosystems) at DNA-lab (SUS, Malmö, Sweden). Due to the standard size range, the resolved fragments or T-RFs will not exceed size of 600 bases which will cover V1-V3 region. Thus the T-RFLP profile generated in this thesis was from the region flanking V1-V3.

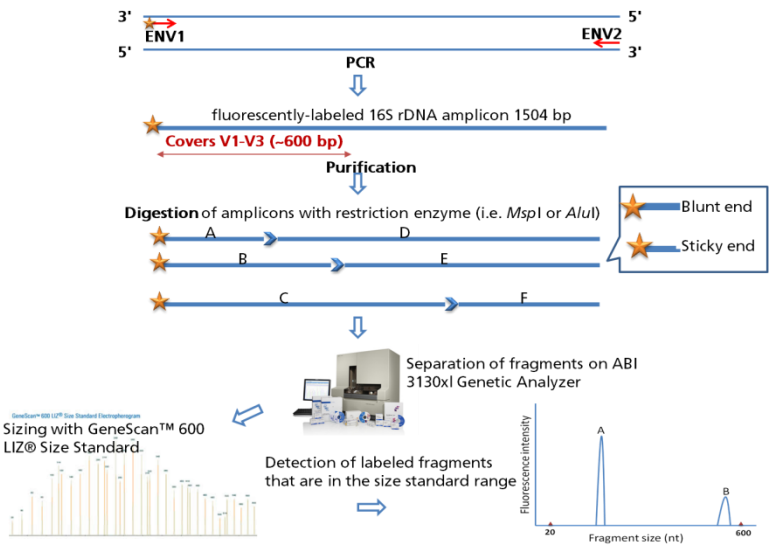

Figure 3. Schematic flow of T-RFLP procedure used in this thesis.

Some technical considerations. T-RFLP involves multiple steps, where optimization is required for each step to produce a reliable result. It is well known that different types of PCR bias may decrease the PCR yield. In practice, we have observed increasing yield of the 16S rDNA amplicons by changing Taq DNA polymerase (Roche Diagnostics, GmbH, Mannheim, Germany) to FastStart Taq DNA polymerase (Roche) then later to TopTaq DNA polymerase (Qiagen). PCR clean-up method is more important when the bacterial DNA is amplified from host tissue (i.e. when the target were mucosa associated bacteria) than from cecal content or feces. At the DNA extraction step, host genomic DNA from tissue will be co-extracted with bacterial DNA and accounts for much higher proportion. In practice, sometimes bright background from the loading well on the gel can be observed together with the target band which indicates the excess DNA present in the PCR solution. Thus simple PCR clean up method may purify the host DNA together with the targeted 16S rDNA amplicons. Gel extraction purification could be better in this case. However, the recovery of this method is about 80%. The loss of the amplicons may introduce bias for further analysis. Later, quantification of purified amplicons with Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, USA) may lead to overestimation of DNA amount.

Some considerations on data analysis. The data generated from T-RFLP were analyzed using Genemapper software version 4.0 (Applied Biosystems, Foster city, CA, USA) with internal size standard GeneScanTM LIZ 600® (range 20-600 bases, Applied Biosystems). Local southern algorism was chosen for size calling. The sizes and areas of detected peaks or T-RFs were used to build the T-RFLP data matrix. Average of the sizes were calculated for the each T-RF (in the same bin), and relative area of every T-RF was calculated by dividing the area of the T-RF with the sum of the area of all the detected T-RFs in a sample. Then, the differences in average sizes of T-RFs smaller than 0.5 bp ~1 bp (decided after evaluating the raw dataset in each study) were regarded as same T-RFs and merged together to generate a new data matrix which was used for subsequent data analysis.

Diversity indices were calculated manually or by using a free online software PAST PAST (version 2.17b; http://folk.uio.no/ohammer/past/). The number of T-RFs was used for evaluating the richness of the microbiota, Shannon's diversity [93] and Simpson's diversity [94] were used for evaluating both the richness and evenness. Sometimes statistical significance was found only in one of the diversity indices which revealed that the major impact was from either the rare species or the dominant species.

Multivariate Data analysis was performed on T-RFLP data matrix using SIMCA-P software (version 12.0.1.0; Umetrics, Umeå, Sweden) to get an overview of the microbiota structure. If clusters were found by Principal Component Analysis (PCA),

partial least squares projection to latent structures (PLS) models were used to find correlations between the microbiota and physiological parameters. Considering the characteristics of the T-RFLP data matrix, which contains many zeros and occasional T-RFs with comparatively smaller abundance, the data set was usually not scaled but centered or sometimes scaled with par scaling [1/sqrt(SD)] in the SIMCA-P scaling options. Unit Scaling was considered not appropriate because the impact of the occasionally detected T-RFs, which may be artifacts or physiologically irrelevant, could be exaggerated.

T-RFLP has the advantage of high reproducibility and T-RFLP data can provide an overview of the microbiota structure in a short time and to a relatively low cost. However, for identifying bacteria that were represented by T-RFs subsequent sequencing techniques are needed.

Quantitative PCR (qPCR)

SYBR green qPCR method was used for bacterial quantification (Fig. 4). Plasmid standard curve was constructed by cloning 16S rDNA fragment that are specific for the target into pGEM-T vector system (Promega, Madison, USA). The following bacterial strains were used for constructing the standards. *Lactobacillus plantarum* DSM 9843 (Deutsche Sammlung von Mikroorganismen, Germany), *Bifidobacterium infantis* DSM15159, *Escherichia coli* CCUG 29300 (Culture collection, University of Gothenburg, Sweden), *Akkermansia muciniphila* DSM 22959, *Clostridium leptum* DSM753, *Faecalibacterium prausnitzii* DSM 17677, *Bacteroides fragilis* ATCC 25285 (American Type Culture Collection) and *Desulfovibrio desulfuricans subsp. desulfuricans* CCUG 34226. The inserts were amplified with the same sets of primers for the amplification of the samples. The standard ranged from 10^2 to 10^8 copies per reaction.

The PCR condition was optimized for each targeted bacterial groups, and the PCR reaction was performed in duplicates or triplicates. Either 2-step (fast cycling by combining annealing and extension into one step) or 3-step qPCR consists of 40 cycles was performed in each study. The amplification specificity was checked by melt curve analysis after each run. Samples were diluted if exceeded the detection limit and rerun. Samples found under the detection limit (<100 copies in 20 ul reaction) were excluded from the data analysis. For the qPCR data interpretation, one should bear in mind that multiple copies of the 16S rRNA genes may exist in some bacterial genome [95], which may lead to an overestimation of the bacterial population.

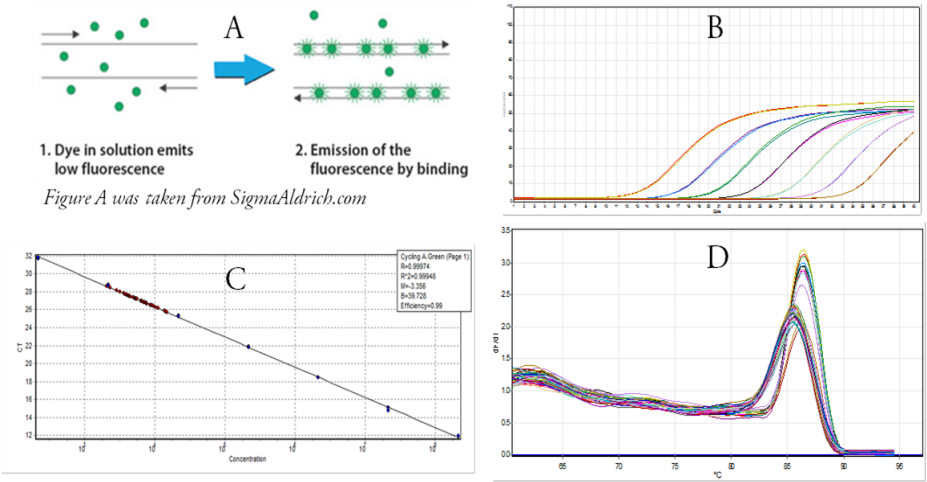

Figure 4. Schematic view of the SYBR green qPCR. A) SYBR green binds to double stranded DNA. B) Amplification curve observed by real-time. C) Quantification with standard curve. D) Melt curve analysis.

Cloning and Sanger sequencing

16S rRNA gene cloning and sequencing was used to identify the T-RFs of interest. The 16S rRNA gene was amplified with unlabeled primers ENV1 (5′-AGA GTT TGA TII TGG CTC AG-3′) and ENV2 (5′-CGG ITA CCT TGT TAC GAC TT-3′). The amplicons were cloned into pGEM-T vector (Promega) and followed by the transformation of *E. coli* JM109 high efficiency competent cells. The procedure of cloning is illustrated in Figure 5. After purification, clones were sent to MWG-Biotech (MWG-Biotech, Ebersberg, Germany) or GATC Biotech (Konstanz, Germany) for Sanger sequencing. The sequencing primer 519r (5′-GWATTACCGCGGCKGCTG-3′) was chosen to cover the ENV1 sequence, therefore the sequences of clones can be used to generate the theoretical length of T-RFs of interest. In addition, cloning was also used to generate plasmid standards for qPCR. Instead of cloning the full length of 16S rRNA gene, fragments of 16S rDNA that were specific for targeted bacterial groups were amplified and cloned into the plasmid vectors.

The drawback of building clone libraries using cloning and Sanger sequencing is that it is laborious and time-consuming.

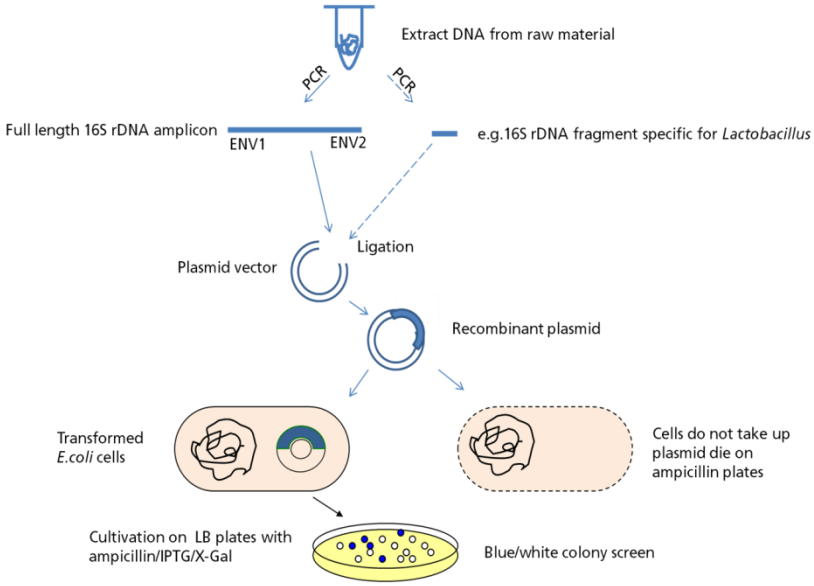

Figure5. Schematic flow of cloning procedure used in this thesis. Either the full length 16S rDNA (solid line) or fragments of 16S rDNA (dashed line) were cloned into plasmid vector.

454 amplicon pyrosequencing

Procedure. 454 amplicon pyrosequencing is a high throughput method which does not require cloning and can rapidly produce sequence data with accuracy. However, the cost is yet high and the method was used on a restricted number of samples in two of the studies in this thesis. To compare with the results from T-RFLP, the same region V1-V3 was targeted (Figure 6). Both forward and reverser primer were barcoded with 10 bases long multiplex identifier (MIDs) from Roche. To identify the direction of the sequences different MIDs were used with forward and reverse primers. The PCR reactions were prepared in triplicates and the amplicons were purified with MinElute Gel Extraction kit (Qiagen). The purified amplicons were quantified with Nanodrop ND-1000 (Saveen Werner, Limhamn, Sweden) and pooled in equal amount. The PCR products were subsequently amplified by emulsion PCR and sequenced at MWG, Germany using GS FLX+ chemistry.

Sequence data analysis was performed using an open source software package Quantitative Insights Into Microbial Ecology (QIIME 1.7.0) [96].

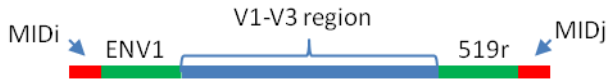

Figure 6. Amplicons used for 454 bi-directional pyrosequencing.

Some considerations on amplicon pool preparation and data analysis methods. To decrease the amplification bias it is important to have equimolar amplicons from different samples. DNA quantification with fluorescence-based technique such as Picogreen assay is usually recommended for higher accuracy in comparison with the Nanodrop spectrophotometer [97]. For the sequence data analysis, there are many ways available. One important thing is that when using script based bioinformatics analysis tools such as QIIME, bash script, verification of each step should be done to avoid obtaining biased results. In practice, gibberish sequence generation, omitted sequences by unknown reason (maybe due to the bug in the code) and different formula for calculating Shannon's diversity index (log base 2 was used in QIIME rather than natural logarithm) have been observed.

The drawback of the pyrosequencing till now could be that shorter sequence length than Sanger sequencing, and the signal drop when there are multiple homopolymers and relatively high cost.

Table 1. Overview of the studies, type of samples, background of diet or treatment and methods used for the microbiota analyses.

Studies	Type of samples	Diet/Treatment	Methods
Paper I	Cecal content of male Sprague Dawley rats	Treatment time: 4 weeks	T-RFLP
		Water+standard chow (n=9)	qPCR
		L-NAME+standard chow (n=9)	
		L-NAME+standard chow+fermented bilberries+HEAL19 (n=9)	
		L-NAME+Standard chow+phenolic acids mix+HEAL19 (n=9)	
Paper II	Cecal content of male Sprague Dawley rats	Treatment time: 4 weeks	T-RFLP
		Water+standard chow (n=9)	qPCR
		Water+fermented bilberries with higher phenolic acid content (n=9)	
		Water+fermented bilberries (n=9)	
Paper III	Mouth swab and fecal samples from hypertensive volunteers	Intervention time: 3 months	T-RFLP
		Placebo (n=30)	454 amplicon pyrosequencing
		Fermented bilberries+HEAL19 (n=28)	
		HEAL19 (n=32)	
Paper IV	Ileal pouch mucosa samples from former patients with UC	Samples were taken one month after surgery (n=2)	T-RFLP
		Samples were taken one year after surgery from the same patient (n=14)	454 amplicon pyrosequencing
Study A	Cecal content of female C57BL/6J (Taconic)	Intervention time: 30 weeks	T-RFLP
		LFD (10% kcal fat) (n=5)	
		HFD (60% kcal fat) (n=5)	
Study B	Cecal content of female C57BL/6NCrl mice (Charles River) and C57BL/6JBomTac mice (Taconic)	Intervention time: 4 weeks	T-RFLP
		Atherogenic diet (energy ratio of 41 % fat) (n=10)	qPCR
		Atherogenic diet supplemented with Oat bran (n=10)	

Study C	Cecal content of female ($LDLr^{-/-}$) mice (Charles River), C57BL/6J is the background strain	Intervention time: 6 weeks High-fat, isocaloric Western diets (n=10) HFD+Purified EPS produced by *Pediococcus parvulus* 2.6 (n=10) HFD+Supplemented with live *P. parvulus* 2.6 (n=9)	T-RFLP Cloning and sanger sequencing qPCR
Study D	Cecal content of male Wistar rats	Intervention time: 6 weeks High fat diet (n=7) HFD+pectin (n=7) HFD+guar gum (n=7)	T-RFLP
Study E	Small intestinal tissue and cecal content of female C57BL/6JBomTac mice (Taconic)	Intervention time: 22 weeks HFD (n=10) HFD+HEAL19 (n=8) HFD+4% green tea (n=10~11) HFD+HEAL19+4% green tea powder (n=11~12)	Culturing RAPD T-RFLP qPCR
Study F	Cecal tissue of female C57BL/6JBomTac mice (Taconic)	Intervention time: 22 weeks HFD (n=10) HFD+HEAL19 (n=10) HFD+HEAL19+0.2% green tea (n=10) HFD+HEAL19+2% green tea (n=10) HFD+HEAL19+4% green tea (n=10)	T-RFLP qPCR

Study G	Cecal content of male Wistar rats	Intervention time: 5 days Controlled feed intake (12g/day, with matched fiber amount) for 5 days (n=7) Supplemented with Blackcurrant (n=7) Supplemented with Blackcurrant+HEAL19 (n=7) Supplemented with Raspberry (n=7) Supplemented with Raspberry+HEAL19 (n=7)	T-RFLP
Study H	Cecal tissue of male Balb/cJ mice	Ischemia-reperfusion sham group with standard chow (n=8) The following 5 groups were fed experimental diets for 10 days and then underwent ischemia-reperfusion I/R control fed standard chow (n=8) Supplemented with bilberry (n=8) Supplemented with bilberry+HEAL19 (n=8) Supplemented with bilberry+*L.plantarum*RESO56 (n=8) Supplemented with bilberry+*Pediococcus acidilactici* JAM046 (n=8)	T-RFLP
Study I	Colonic tissue of female C57BL/6 mice (Charles River)	4% DSS was given for 7days. Non treated health mice (n=10) Mice with DSS-induced colitis (n=10)	T-RFLP qPCR

For the abbreviations please refer to page 9.

Results and Discussion

In the following, results are referred and discussed from the four papers included in this thesis, but also nine other studies where the author has performed the microbiota analyses are discussed. The multitude of different studies is justified by the use of T-RFLP throughout all the studies and the unprejudiced search for health-related, universal bacterial taxa affected by dietary compounds.

It should be noted that all the T-RFs compared in this thesis are from *Msp*I digestions.

Part I. Response of the gut microbiota to dietary components

Study A (Olsson et al.): HFD or LFD to mice

Ten C57BL/6J mice were divided into two groups, one group fed low fat diet (LFD, 10%kcal fat) and the other group fed high fat diet (HFD, 60%kcal fat) for 30 weeks. Cecal microbiota was analyzed with T-RFLP. No significant difference was found in richness (measured by number of detected T-RFs), dominance or Shannon's diversity index. However, a shift in the bacterial composition was observed by PCA analysis (Fig. 7). The major change was that abundance of T-RF 117 ("*Allobaculum*-like") decreased in HFD fed mice compared to LFD fed mice (means 10% vs. 3%), whereas T-RF 265 ("*Akkermansia*-like") was increased in HFD (means LFD vs. HFD, 13% vs. 26%). Significantly increased *Akkermansia* load was also confirmed by comparing clone libraries (data not shown). This is opposite from the observation by Everad et al. [49], where they also used 60% fat high fat feeding and saw a decreasing abundance of *Akkermansia*. The other abundant T-RFs suppressed by HFD feeding were T-RF 88, T-RF 90 and T-RF 271.

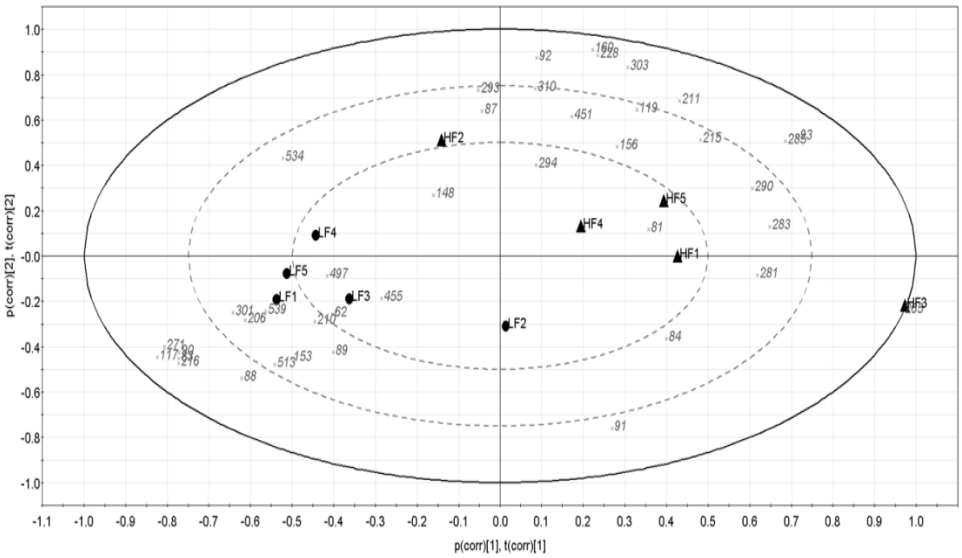

Figure 7. PCA loadings bi plot based on T-RFLP data explained 47.3% total variance (31.6% by PC1, 15.7% by PC2). Dots: low fat diet fed mice (n=5); triangles: high fat diet fed mice (n=5). Numeric numbers in grey colors represent sizes of detected T-RFs. *From study A (Olsson et al., 2013).*

Study B (Andersson et al.): Oat bran supplementation to mice:

The cholesterol lowering effect by oat bran was observed in C57BL/6NCrl mice but not in C57BL/6JBomTac mice. The two mice strains were purchased from different suppliers. Shannon's diversity index was not significantly affected in C57BL/6Ncrl mice (median control vs. oat bran: 2.0 vs. 2.1) whereas the diversity index was significantly reduced in C57BL/6JBomTac mice by the oat bran supplementation (median control vs. oat bran: 2.5 vs. 1.7). Comparison between the two control groups of the two substrains showed that C57BL/6JBomTac mice had a significantly more diverse cecal microbiota. qPCR results showed that oat bran supplementation significantly increased the total load of bacteria, and the amount of *Akkermansia* and "*Bacteroides fragilis* group" in both substrains. A significant interaction between the oat bran treatment and the substrains was also observed for *Akkermansia*. PCA analysis on T-RFLP data was carried out to get an overview of the microbiota in the two substrain groups and OPLS regression model was used to further investigate the correlation between the microbiota and cholesterol metabolism. The first two principal components explained 34.9% and 14.3% of the variance of the T-RFLP data respectively. Four groupings were seen in the PCA analysis, indicating different structure in the cecal microbiota between the substrains and also between the

different diet groups in the same strain. The PLS analysis in Figure 8 shows a link between the microbiota change and markers of cholesterol metabolism. The same control diet shaped the cecal microbiota in the two mice substrains differently. Moreover, the oat bran supplementation altered the cecal microbiota in different direction in the two substrains. For instance, T-RF 271 was only detected in oat bran fed mice of C57BL/6JBomTac strain and this group of mice had an increased abundance of T-RF 283. It has been shown that host genetics, and housing environment can affect the microbiota structure [98-100]. The different response to the same diet observed in study B could have originated from both genetics and environmental factors. The plasma cholesterol lowering effect of oat bran consumption seen in the C57BL/6NCrl mice was mainly due to the increased excretion of the bile acids. Interestingly, the increase in the abundance of T-RF 93 ("*Bacteroides*-like") was only seen in the C57BL/6NCrl substrain. Many species of *Bacteroides* are able to deconjugate bile acids [101]. McKnite et al. found that different quantitative trait loci (QTL, DNA regions containing the genes that are associated with the phenotypes) could separately modulate the population of *Bacteroides*, *Rikenellaceae*, *Prevotellaceae* [98]. The different responses of the gut microbiota of the two mice substrains observed in **study B** also suggest that the outcome of a diet intervention may be influenced by the variation in the gut microbiota of the murine models.

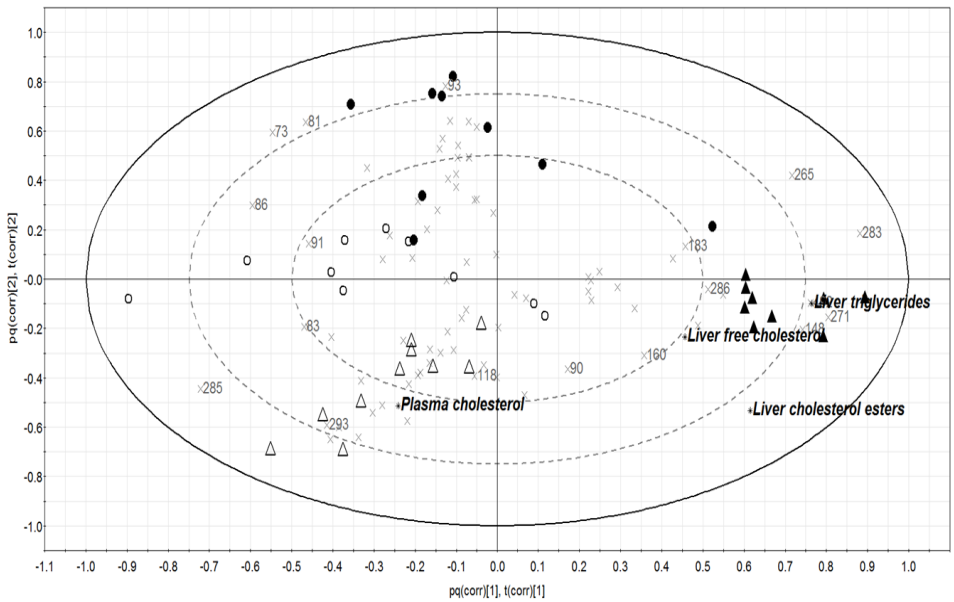

Figure 8. Orthogonal partial least squares projections to latent structures (OPLS) loadings bi plot based on data matrix of T-RFLP and markers of cholesterol metabolism. Circles: C57BL/6NCrl control mice; dots: C57BL/6NCrl oat bran fed mice; open triangles: C57BL/6JBomTac control mice; filled triangles:

C57BL/6JBomTac oat bran fed mice. Numeric numbers in grey colors represent sizes of detected T-RFs. *From study B (Andersson et al., 2012).*

Study C (Lindström et al.): EPS or EPS producing bacteria to mice:

Purified exopolysaccharide (EPS) produced by *Pediococcus parvulus* 2.6 was given as supplements to high fat diet to mice with C57BL/6J background in order to evaluate the prebiotic effects. However, instead of showing the suggested bifidogenic effect [102] EPS suppressed the growth of bifidobacteria and significantly decreased cecal bacterial diversity. In contrast, the supplementation of live *Pediococcus parvulus* 2.6 did not reduce the diversity but antagonized *Enterobacteriaceae*. In addition, qPCR results showed that *Akkermansia* were significantly increased in both supplemented groups and the "*Bacteroides fragilis* group" was significantly increased in the EPS supplemented group when it was compared to the *P.parvulus* 2.6 supplemented group, but not to the control. The effect of the supplementation on the cecal microbiota composition was analyzed with PCA (Fig. 9). The first two principal components together explained 33% of the total variance. PC1 (19% of the variance) explained the effect of pure EPS supplementation in comparison to the other three diets. The main driving forces for the EPS grouping were T-RF 118 and T-RF 491. PC2 (14% of the variance) mainly explained the effect of live *P. parvulus* 2.6 supplementation in comparison to the control diet without supplementation.

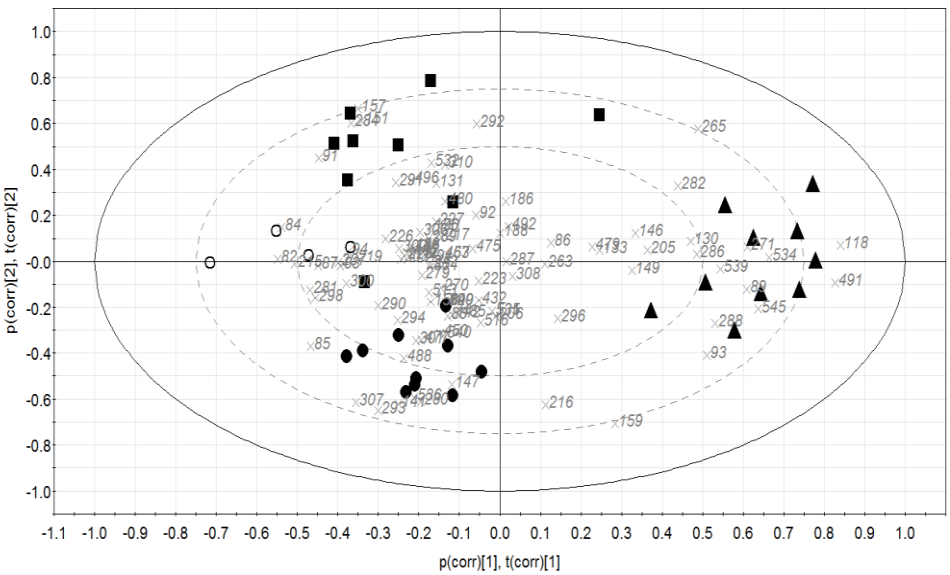

Figure 9. PCA loadings bi plot based on T-RFLP data. Circles: baseline mice did not receive any experimental diets (n=4); Dots: mice fed control diet (high fat); triangles: mice given pure EPS supplementation to food; boxes: mice given live EPS producing *Pediococcus parvulus* 2.6 (10^8 CFU/g) as

supplementation to food. Numeric numbers in grey colors represent sizes of detected T-RFs. *From study C (Lindström et al., 2013).*

Study D (Jakobsdottir et al.): Fibers to rats:

The cecal microbiota of Wistar rats eating high fat diet for 6 weeks with fiber-free, pectin or guar gum supplemented diet was analyzed by T-RFLP. The supplementation did not significantly affect the bacterial diversity when estimated with Shannon's diversity index (medians control vs. guar gum vs. pectin: 1.97 vs. 2.4 vs. 2.05). However, the fiber supplementation shifted the microbiota composition in different ways. T-RF 88 was found with average abundance of 6% in the control group, but was only detected in two mice from the fiber supplemented groups. T-RF 155 was found with an average abundance of 5% in control but occasionally found in the fiber supplements groups with average abundance lower than 1%. The dominant T-RF 265 (*Akkermansia*-like) showed a sharp drop in abundance by fiber feeding (means, control vs. guar gum vs. pectin: 37% vs. 5% vs. 9%). Two types of fiber also exerted different selection pressure towards the cecal microbiota as shown by PCA analysis (Fig. 10). The first two principal components explained 59% of the total variance (26.8% by PC1 and 32.2% by PC2). T-RF 220 was highly boosted by pectin, the average abundance accounted for 28%, but it was only detected in one mouse from the control group and was totally absent in the guar gum fed mice. Comparing pyrosequencing result from **paper IV**, the putative ID of T-RF 220 is *Lachnospiraceae*. Species belonging to this family, for example, *Lachnospira multiparus* are known to grow well on pectin [103, 104]. T-RF312 also showed a similar trend, it had average abundance of 17% in pectin fed mice, but was absent in the control group and was only detected in one mouse from guar gum group. The T-RF 94 (6%) was only detected in the pectin fed group. The pectin fed animals had significantly less weight gain than the control group. On the other hand, guar gum favored the growth of taxa represented by T-RF 84, T-RF 87, T-RF 92 (20%), T-RF 93 (7%), T-RF 221 (9%) and T-RF 542 (5%). T-RF 221 and T-RF 542 were absent in the control and pectin group.

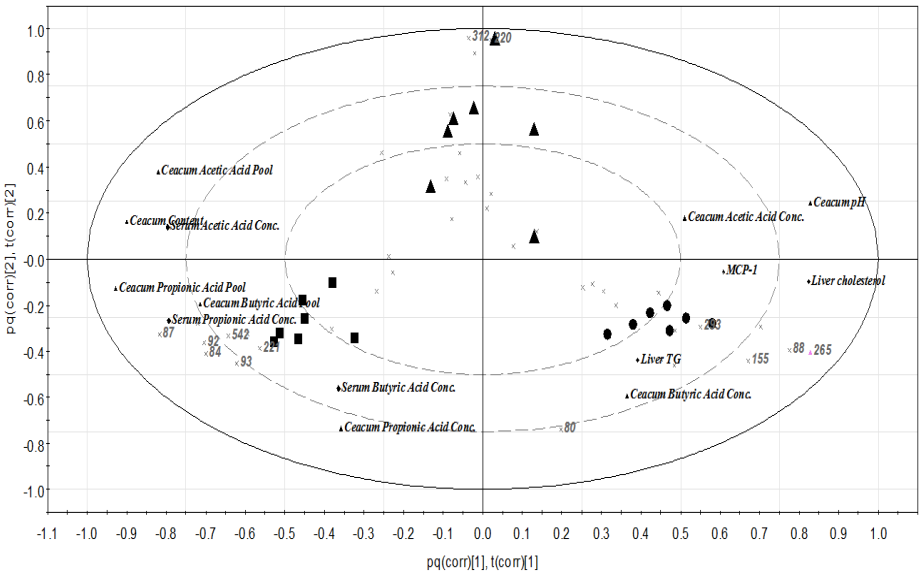

Figure 10. PLS loadings bi plot based on the cecal microbiota and other analyzed parameters. Dots: rats fed fiber-free high fat diet; triangles: rat fed pectin as supplementation; boxes: rats fed guar gum as supplementation. Numeric numbers in grey colors represent sizes of detected T-RFs that have most influence for the clustering. From study D (Jakobsdottir et al., 2013).

Study E (Axling et al.): Green tea and/or HEAL19 to mice:

Small intestinal and cecal microbiota of C57BL/6JBomTac mice fed high fat diet supplemented with *Lactobacillus plantarum* HEAL19 (Lp), 4% green tea powder (GT) or the combination of the two (Lp+GT) were analyzed with T-RFLP and/or qPCR. After 22 weeks, the bacterial diversity in the small intestine was significantly increased in the Lp+GT group compared to the control and the GT group, whereas no significant difference was found in cecal bacterial diversity. Quantification of certain bacterial groups in small intestine showed that Lp+GT had significantly more *Lactobacillus* compared to the control and the GT group. No significant difference was found in either total amount of bacteria or in the load of *Akkermansia*. However, there was a link between shift in bacterial composition and physiological parameters as shown by PLS analysis (Fig. 11). For small intestinal microbiota, the first two principal components explained 25.6% (PC1) and 15.6% (PC2) of the total variance respectively. For cecal microbiota, the first two principal components explained 26.4% (PC1) and 16.4% (PC2) of the total variance respectively. For the small intestinal microbiota, control group (HFD) was enriched with T-RF 118 and T-RF 184, whereas these taxa were suppressed by the supplementations. T-RF 568 represents HEAL19, which was found increasing in abundance with the supplementation both in the small intestine and cecum. Green tea supplementation boosted taxa represented

by T-RF 270, T-RF 271 and T-RF 307 in the small intestine and cecum. Larger individual variation in Lp group was seen in the small intestine in comparison to cecum. T-RF 497 was enriched in control and Lp group but suppressed by GT and Lp+GT supplementation in both small intestine and cecum.

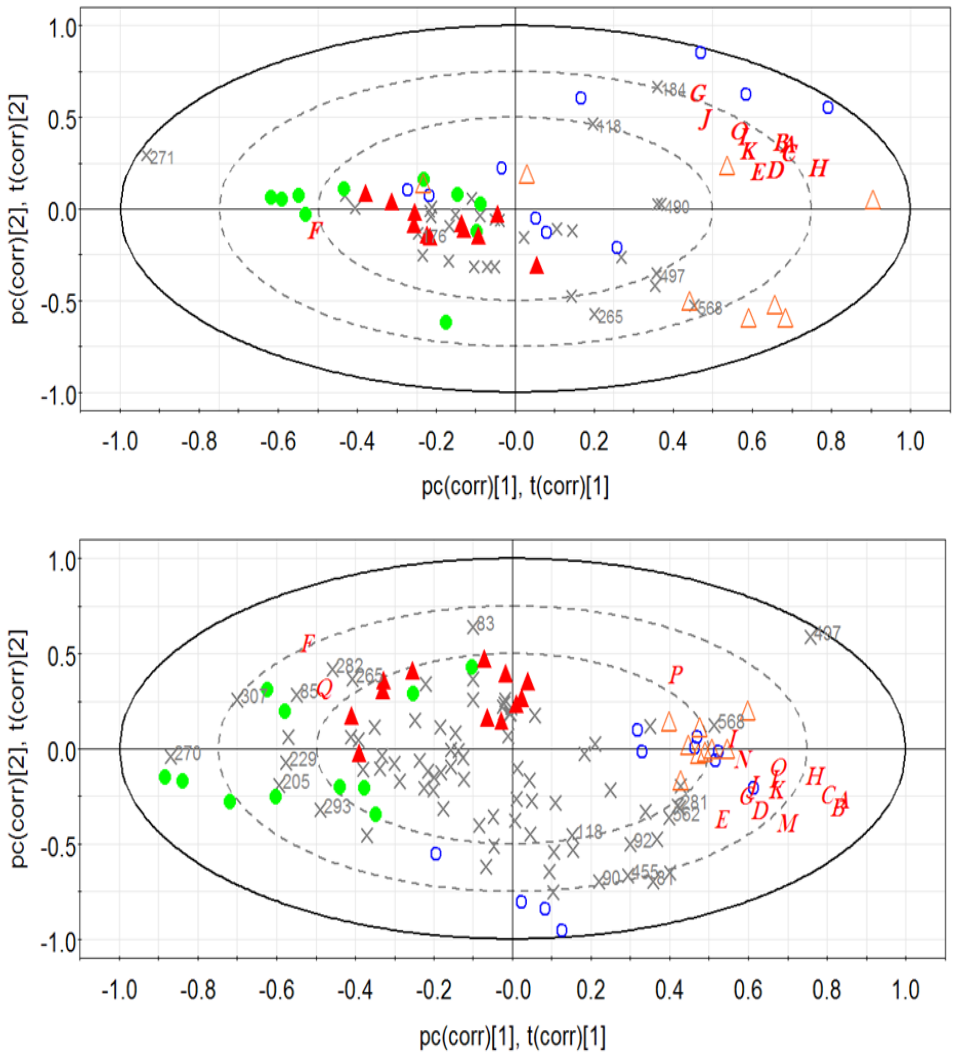

Figure 11. PLS loadings bi plot based on the cecal microbiota and other analyzed parameters. Up-figure: Small intestine; Down-figure: Cecum. Circles: mice fed high fat diet; open triangles: mice fed live *L. plantarum* HEAL19 as supplementation (Lp); green dots: mice supplemented with green tea (GT); red triangles: mice fed the combination of *L. plantarum* HEAL19 and green tea (Lp+GT). Numeric numbers in grey colors represent sizes of detected T-RFs that have most influence for the clustering. Letters are measured physiological parameters. A, body weight; B, body fat content; C, periovarian white adipose

tissue; D, liver weight; E, spleen weight; F, caecum weight; G, liver cholesterol; H, liver TAG; I, plasma ALT; J, plasma cholesterol; K, plasma insulin; L, plasma glucose; M, plasma leptin; N, PPARα mRNA; O, PPARγ mRNA; P, CD36 mRNA; Q, SR-B1 mRNA. *From study E (Axling et al., 2012).*

Study F (Xu et al.): HEAL19+different doses of green tea to mice:

The effects of different doses of green tea on the cecal microbiota when combined with live *L. plantarum* HEAL19 were investigated. C57BL/6JBomTac Mice were fed high fat diet for 22 weeeks with or without the following supplmentation: *L. plantarum* HEAL19 alone (L); L combined with 0.4% green tea powder (L0.4G); L combined with 2% green tea powder (L2G); L combined with 4% green tea powder (L4G). The amount of *Lactobacillus* was significantly increased in all the supplemented groups compared to the control group. L4G had the least increase in the *Lactobacillus* amount and it was significantly lower than L group. The green tea powder at lower doses 0.2% and 2% induced highest increase in bacterial diversity while only 4% green tea suppressed the growth of *Enterobacteriaceae* and shifted the microbiota composition to the highest degree (Fig. 12). The first two principal components explained 23.1% (PC1) and 13.1% (PC2) respectively. T-RF 117 and T-RF119 were decreased with supplementations, while T-RF 294, T-RF 497, T-RF 88 were increased in abundance in green tea dose dependent manner. In addition, no significant difference was found in the amount of bifidobacteria, *Akkermansia*, *Desulfovibrio* and *"Clostridium leptum* group*"* between groups.

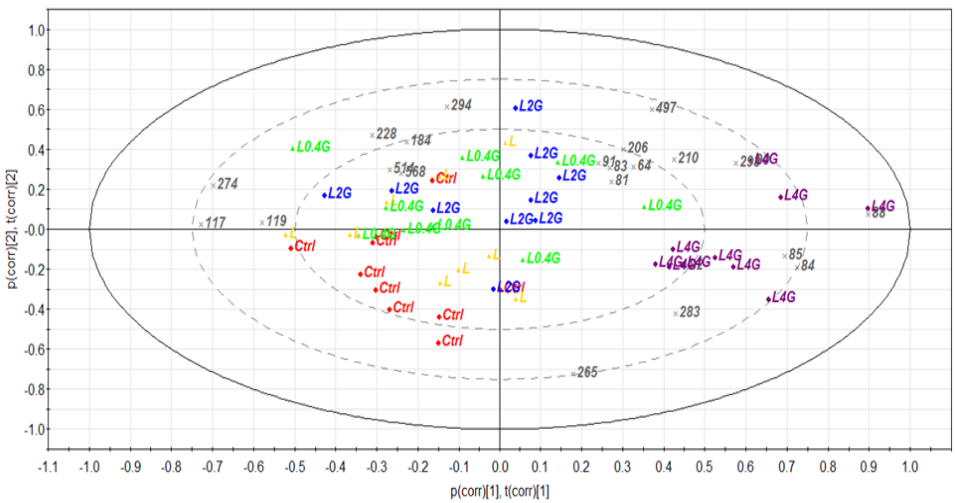

Figure 12. PCA loadings bi plot based on T-RFLP data. Ctrl: mice fed control diet (high fat); L: mice supplemented with *L.plantarum* HEAL19 ; L0.4G: mice supplemented with HEAL19 and 0.4% green tea; L2G: mice supplemented with HEAL19 and 2% green tea; L4G: mice supplemented with HEAL19

and 4% green tea. Numeric numbers in grey colors represent sizes of detected T-RFs. *From study F (Xu et al., 2013).*

Study G (Jakobsdottir et al.): Berries with/without HEAL19 to rats:

The T-RFLP data of cecal microbiota was re-anlayzed from the original data in this thesis to show the contributing T-RFs so that can be compared with other studies. Wistar rats were given freeze-dried berry diets alone or together with *L.plantarum* HEAL19. Due to the DNA extraction failure from the blackberry group, cecal microbiota analysis was performed on the groups receiving blackcurrant or raspberry. Addition of *L.plantarum* HEAL19 did not significantly increase the bacterial diversity within the same berry group. However, comparison between the groups that only received berries showed that the raspberry group had significantly higher diversity than the blackcurrant group. PCA analysis of the T-RFLP (Fig. 13) showed that the addition of the *L.plantarum* HEAL 19 did not led to an obvious change in the composition of the cecal microbiota. The first two principal components explained 23.8% (PC1) and 14% (PC2) of the total variance respectively. The two berries favored different bacterial groups. Raspberry mainly boosted the growth of taxa reprensented by T-RF 131, T-RF 294, T-RF 196 whereas blackcurrant favored T-RF 86 and T-RF 90-93. In addition, the rats fed blackcurrant showed larger individual difference.

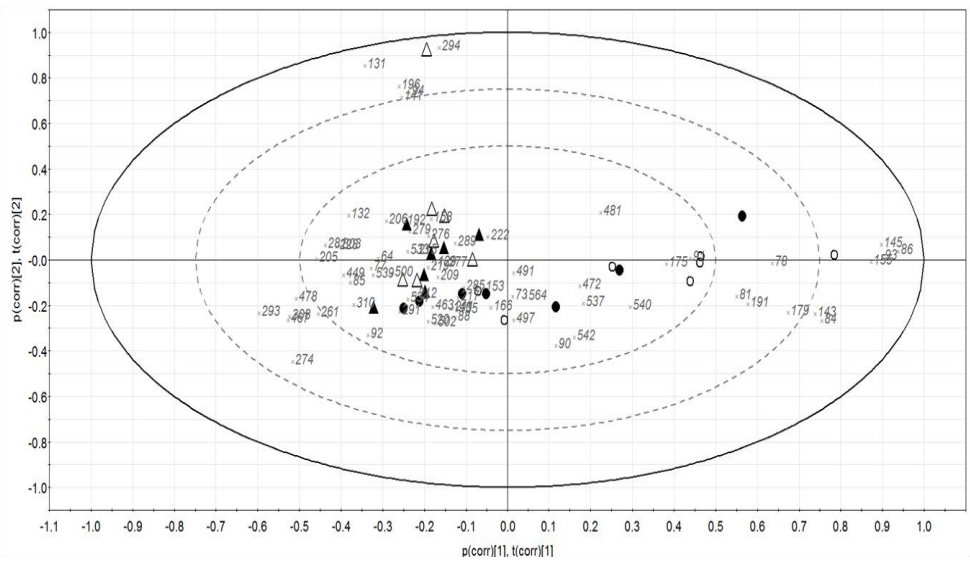

Figure 13. PCA loadings bi plot based on T-RFLP data. Circles: rats supplemented with blackcurrant; dots: rats supplemented with blackcurrant and HEAL19; open triangles: rats supplemented with raspberry; filled triangles: rats supplemented with raspberry and HEAL19.Numeric numbers in grey colors represent sizes of detected T-RFs. *From study G (Jakobsdottir et al., 2013).*

Study H (Jakesevic et al., 2013): Bilberries with/without probiotics to ischemia-reperfusion-mice:

The T-RFLP data of the cecal microbiota was re-anlayzed from the original data in this thesis to show the contributing T-RFs in order to be able to compare with other studies. No animal was excluded here. The Balb/cJ mice were put on different diets and then subjected to surgery in order to create ischemia-reperfusion in the intestine. Mice were divided into 6 diet groups. The diet consisted of standard chow (R3; Lactamin, Stockholm, Sweden) with or without the following supplementations: I/R-control and I/R-sham groups fed standard chow; B group received bilberry powder supplmentation; B+Ped group received bilberry together with *Pediococcus acidilactici* JAM046 strain; B+Lpl56 group received bilberry together with *Lactobacillus plantarum* RESO56 strain; B+LplH19 group received bilberry together with *L.plantarum* HEAL19. The experimental diets were fed for 10 days. The cecal bacterial diversity measured by Shannon's diversity index did not show significant difference between groups. PCA analyis of T-RFLP data matrix (Fig. 14) showed that the bilberry altered cecal microbiota composition whereas the addition of the living bacteria did not have an obvious impact. The first two principal components explained 30% (PC1) and 12.4% (PC2) of the total variance. T-RF 81 and T-RF 206 were most abudant in I/R-sham and I/R control groups, and T-RF 201 and T-RF 293 were only detected in these two groups. On the other hand, T-RF 84, T-RF 92, T-RF90 had higher abundance in the mice received supplmentations. T-RF 265 ("*Akkermansica*-like") was not detected in I/R-sham and I/R control groups. The abundance of T-RF 88 and T-RF 497 were highest in B+ped group.

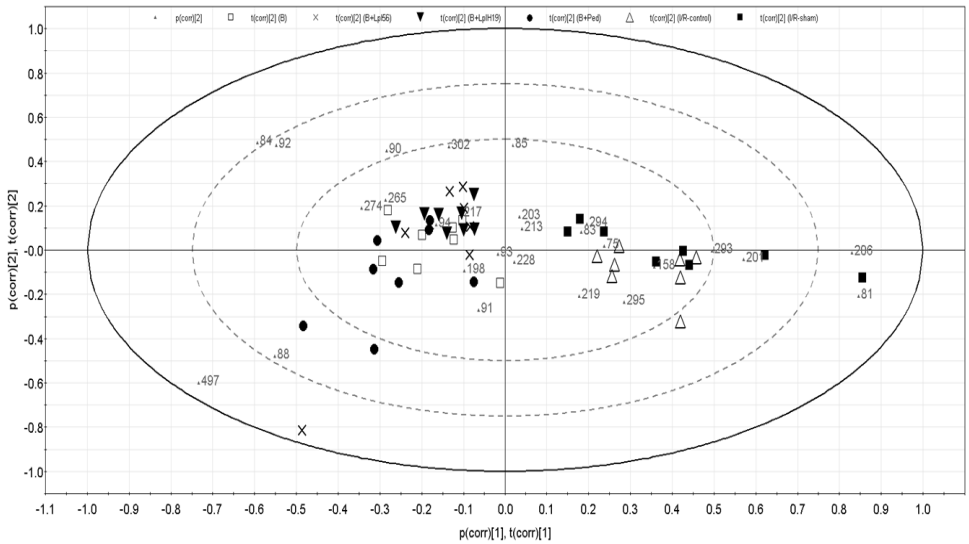

Figure 14. PCA loadings bi plot based on T-RFLP data. Black boxes: mice in I/R-sham group; open triangles: mice as I/R control; open boxes: mice supplemented with bilberry; dots: mice supplemented with bilberry+*Pediococcus acidilactici* JAM046; black inverted triangles: mice supplemented with bilberry+HEAL19; diagonal crosses: mice supplemented with bilberry+*L.plantarum* RESO56. Numeric numbers in grey colors represent sizes of detected T-RFs. *From study H (Jakesevic et al., 2013).*

Paper I. HEAL19 with fermented bilberries or phenolic mixture to L-NAME-rats:

In this study, Sprague Dawley rats were given N_{ω}-Nitro-L-arginine methyl ester (L-NAME) in the drinking water to induce hypertensive state. Rats were divided into 4 groups. The control group was fed standard rodent chow and not treated with L-NAME. L-NAME control group was treated with L-NAME and fed standard rodent chow. LN+FBL group was treated with L-NAME and fed experimental diet supplemented with *L.plantarum* HEAL19 (10^9 CFU/animal/day) and fermented bilberries by the same bacterial strain. LN+PML group was treated with L-NAME and fed experimental diet supplemented with HEAL19 and mixture of phenolic compounds that were found in fermented bilberries. Cecal microbiota was analyzed with T-RFLP and qPCR. The bacterial diversiy was not significantly affected by either L-NAME or the supplementations. However, L-NAME alone conferred selection pressure on the cecal microbiota, a shift in the bacterial compositon was observed by PCA analysis (Fig. 15). Intrestingly, this selection pressure was elimiated by the combination of the three phenolic compounds found in blueberries fermented with *L. plantarum* HEAL19. On the other hand, L-NAME with blueberries fermented with *L.plantarum* HEAL19 altered the cecal microbiota to different degrees in different individual rat. T-RF 303 was most abundant in non-L-NAME treated rats (averge 15%) but decreased with L-NAME treatment (LN group, 3%). PML

supplementation did not have profound impact on T-RF 303 (LN+PML group, average 12%), but FBL supplementation decreased the abudance of this taxa in higher degree (LN+FBL group, average 7%). T-RF 93 was most abundant in LN group (average 16%) and least abundant in LN+FBL group (5%). On the other hand, T-RF 88 was most abudent in LN+FBL group (average 19%) compare to the other three groups (average 4-5%). T-RF 265 was not very different between groups, the average abundance were 4%, 4%, 5% and 8% for control, LN, LN+FBL and LN+PML respectively. qPCR result showed that the amount of *Lactobacillus* was significatnly decreased, whereas "*Bacteroides fragilis* group" was significantly increased in LN+FBL compared to LN. No significant difference was found in the load of total bacteria, *Enterobacteriaceae* or *Akkermansia*.

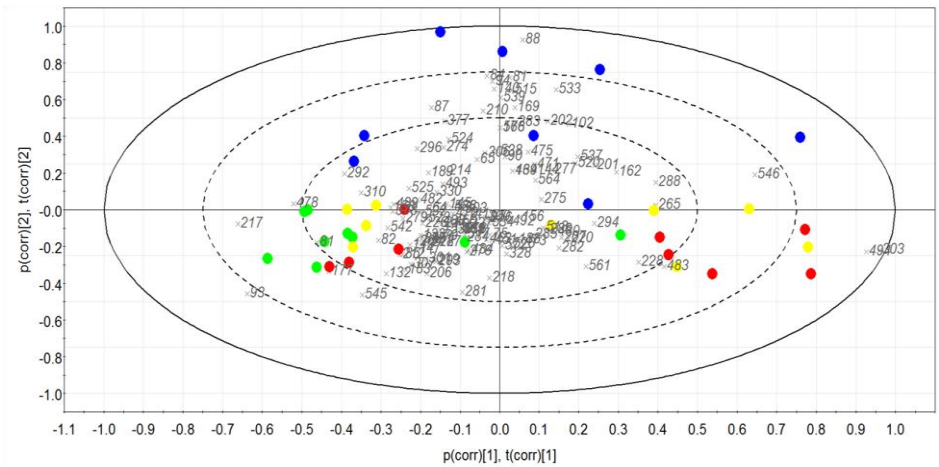

Figure 15. PCA loadings bi plot based on T-RFLP data. PC1 and PC2 explained 31.1% and 17.7% of the total variance respectively. Red dots: non-L-NAME treated healthy control rats ; green dots: L-NAME treated rats; yellow dots: L-NAME treated rats supplemented with HEAL19+phenolic mixture; blue dots: L-NAME treated rats supplemented with fermented bilberry+HEAL19. Numeric numbers in grey colors represent sizes of detected T-RFs. *From paper I.*

Paper II. Fermented bilberries to healthy rats:

In this study, Sprague Dawley rats were randomly divided into six groups, three goups were treated with L-NAME to induce hypertension. Two study products were given to the rats to investigate the anti-hypertensive potential. Product A was femented biberries with higher phenolic acids content and Product B was fermented bilberries. Both products were feremented by HEAL19. Live HEAL19 was also added to the feed. The blood pressure lowering effect of product A was observed in healthy rats. Cecal microbiota of these healthy rats were analyzed to see the effects of the products. Shannon's diversity index was not sigfniciantly different between the groups,

however a shift in microbiota and a cage-effect were observed (Fig. 16). T-RF 265 ("*Akkermansia*-like") was not dectected in this study. Rats fed the experimental diets had different cecal microbiota when compared to the control group. However, the difference was not obvious between the two groups receiving the supplementations. T-RF 303 was most abudent in the control group (average 11%) and decreased in abudance with product A and product B supplementations (4% and 2% respectively). T-RF 132 was absecent in the control group (W), but found with average abundance of 8% in product A fed group (W+A) and 5% in product B fed group (W+B). T-RF 88, T-RF 91, T-RF 92 increased moderately in abudance in the W+A and W+B groups.

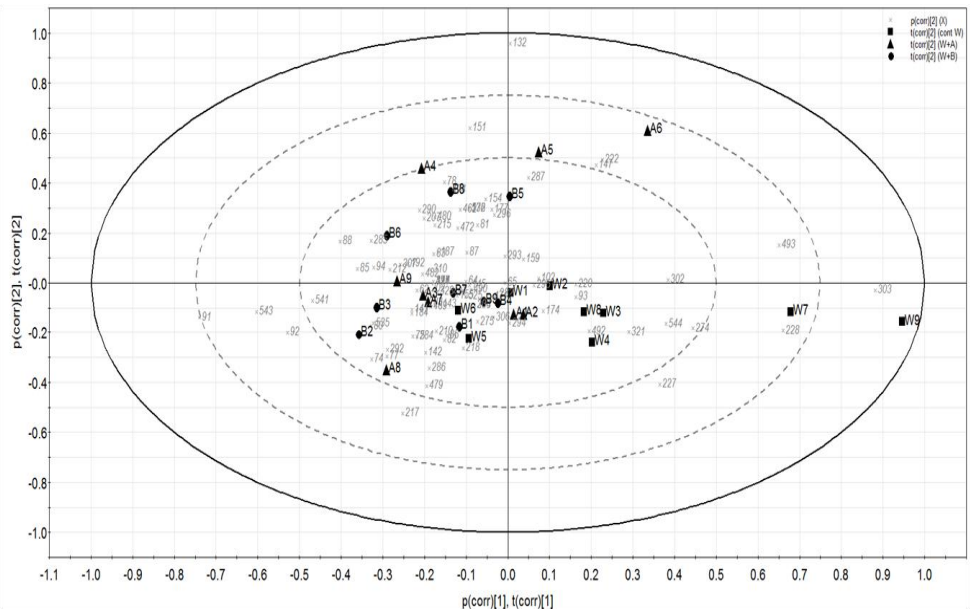

Figure 16. PCA loadings bi plot based on T-RFLP data. PC1 and PC2 explained 27% and 18.9% of the total variance respectively. Boxes: non-L-NAME treated control rats with nothing added in the drinking water; triangles: non-L-NAME treated rats supplemented with HEAL19+product A (higher phenolic acids content than product B); dots: non-L-NAME treated rats supplemented HEAL19+product B (fermented whole bilberry). Rat numbered 1-3, 4-5 and 7-9 were separately housed in the same cage (3 rats/cage). Numeric numbers in grey colors represent sizes of detected T-RFs. *From paper II*

Paper III. Placebo, HEAL19 or fermented bilberries+HEAL19 to hypertensive research persons:

Volunteers with high blood pressure (BP) were randomly divided into three groups and given placeo, HEAL19, or HEAL19+fermented bilberries (fermentation done by

the same bacterial strain) as dietary supplments for 3 months. The studied products did not lower BP during the study period. No obvious effects from the studied supplements were seen on the oral and fecal microbiota. The oral and fecal microbiota were relatively stable, but the oral microbiota fluctuated more with time and showed a much larger individual variation. *Bacteroides* and unclassified *Rikenellaceae* were found to be the two most fluctuating major taxa in the fecal microbiota. *Akkermansia* was detected in feces of 43 out of 90 individuals and showed large variation between individuals regarding the change in abundance by time. In addition, no distinct enterotypes could be identified. Some T-RFs could be putatively identified by matching the results from pyrosequencing (Fig. 17)

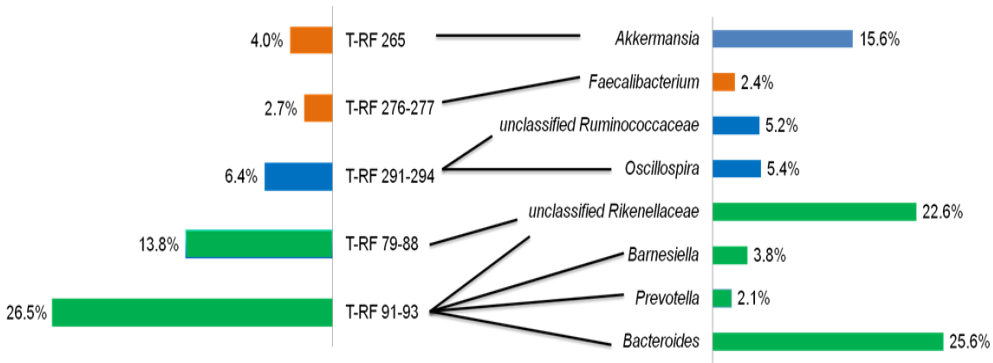

Figure 17. Identification of certain T-RFs by matching T-RFLP and pyrosequencing results. *From paper III.*

Part II. Gut microbiota in the ulcerative colitis

Study I (Håkansson et al.): mice with DSS-induced colitis:

Colitis was induced in C57BL/6 mice by oral administration of 4% dextran sodium sulfate (DSS) for 7 days. The microbiota associated to the colonic musosa was analyzed with T-RFLP and qPCR. Shannon's diversity index was not significantly different between the colitis group and healthy control. However, a shift in microbiota composition was observed (Fig. 18). Furthermore, qPCR results showed that the total load of bacteria significantly increased in the colitis group and so did the load of *Akkermansia* and *Desulfovibrio.* In contrast, *Lactobacillus* was significantly reduced in the colitis group compared to the healthy control group. The prevelence of

Enterobacteraiceae was significantly higher in the colitis group. Only one mouse out of nine mice had *Enterobacteriaceae* in the detection range in the healthy control group.

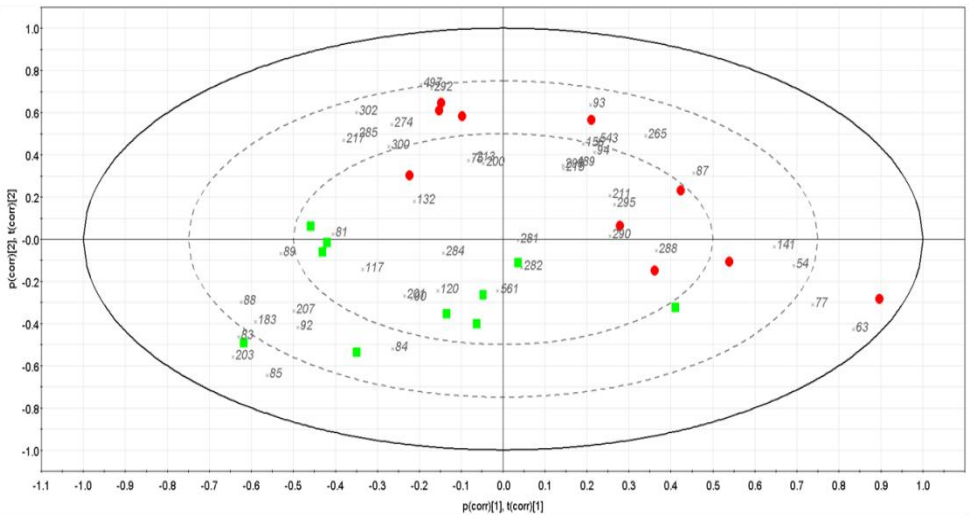

Figure 18. PCA loadings bi plot based on T-RFLP data. PC1 and PC2 explained 17.5% and 16.5% of the total variance respectively. Green boxes: non-DSS treated healthy control mice; red dots: mice with DSS-induced colitis. Numeric numbers in grey colors represent sizes of detected T-RFs. *From study I (Håkansson et al.)*

Paper IV. Ileal pouch microbiota from former UC patients:

Ileal pouch mucosal samples were collected one month and one year after surgery. The ileal pouch microbiota was analyzed with T-RFLP and pyrosequencing. 16S rDNA amplification was only successful with two samples taken at one month after surgery. An optimization of PCRs did not result in increased yield of amplicons that were enough for further analysis. Large individual variation was observed in T-RFLP pattern. The shannon's diversiy index measured on T-RFLP data from the samples taken one year after surgery ranged between 0.5 and 2.5. Pyrosequencing result showed that the ileal pouch mucosa samples taken at ten months after the surgery was predominated by *Firmicutes* (92%) and followed by *Bacteroides* (4%) and *Proteobacteria* (3%) at phylum level (Fig. 19). The ten most abudant genera at both sampling time points were *Clostridium, Blautia, Roseburia, Lachnospira,* unclassified *Lachnospiraceae*, *Bacteroides, Faecalibacterium,* unclassified *Petostreptococcaceae* and *Megamonas* (Fig. 20). qPCR results showed that in the samples taken one month after

surgery, only patient number one had the amount of *F.prausnitzii* to land in the detection range. In patients 13 and 14, *F.prausnitzii* was not detected at any of the two time points. The amount varied between individuals and the largest difference was around 100 fold.

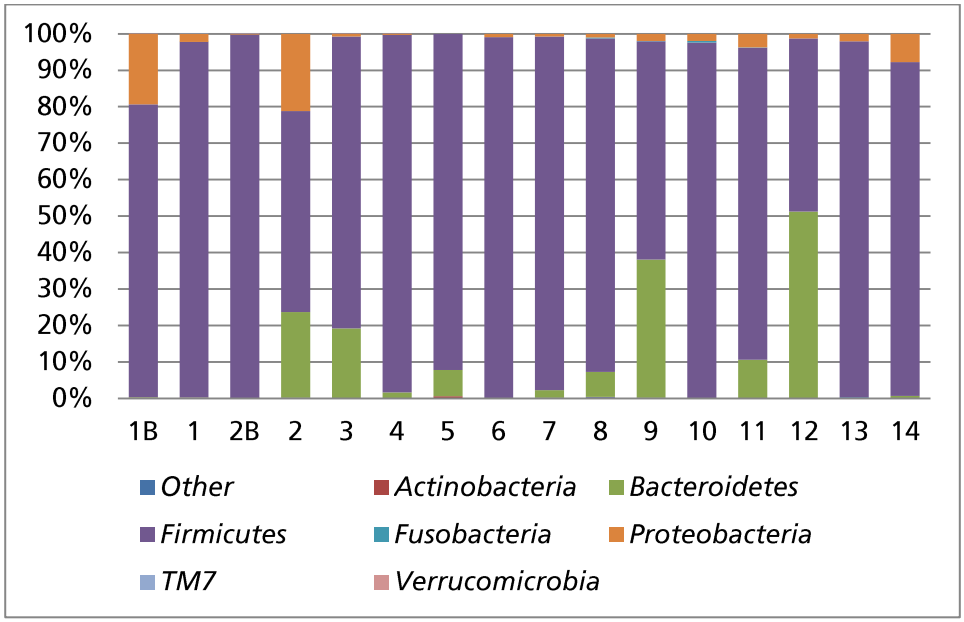

Figure 19. Ileal pouch microbiota composition of former UC patients at phylum level using pyrosequencing. Patients were numbered 1-14. B denotes sample taken one month after surgery. *From paper IV*.

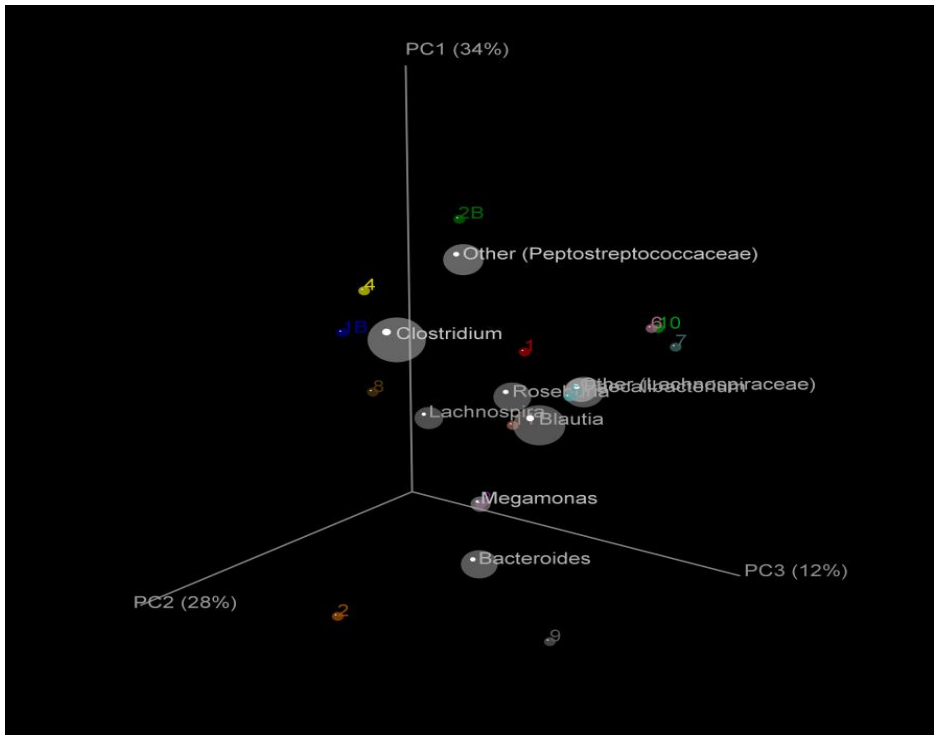

Figure 20. 3D Bi-Plot of the principal coordinate analysis on weighted UniFrac distance matrix. The ten most abundant taxa at genus level were plotted with circles proportional to their abundance. Patients were numbered 1-11. Patients 12-14 were excluded due to the low number of sequences (<500). B denotes sample taken at one month after surgery. *From paper IV.*

Comparison of the results

The driving forces of the clustering in the PCA analysis of T-RFLP data in the above mentioned studies were the dominant T-RFs with high abundance. This leads to the question, are there certain T-RFs responding the same way to the high fat diet or dietary supplementations?

Putative identifications were done by combining T-RFLP and cloning and sequencing techniques including running T-RFLP on the candidate clones and *in silico* digestion using extracted sequences either from RDP or pyrosequencing results.

T-RFs 80-88 could be generated by the family *Rikenellaceae* and the genus *Parabacteroides*. These T-RFs were detected commonly in both murine models and

human samples. Unfortunately, not all of these T-RFs could be identified. *Rikenellaceae* have been reported to be one of the most abundant bacterial taxa in the large intestine of mice and rats [105, 106]. Some interesting trends of these T-RFs were observed in different studies of this thesis. T-RF 88 which can be generated from members of *Rikenellaceae* or *Parabacteroides* were proliferated with polyphenol rich green tea (**study E** and **F**) and bilberry supplementation (**paper I** and **II**). It seemed that this group of bacteria tolerated high fat feed, but were affected by dietary fibers. In **study D**, both pectin and guar gum supressed T-RF 88, whereas in fiber free high fat control it was detected with average abundance of 6%. On the other hand, T-RF 84 and 87 were increased mainly in guar gum fed rats. Rats fed low bush wild bluberry (*Vaccinium angustifolium*) enriched diet was shown to have increased proportion of *Rikenelllaceae* [106]. In **study I**, members of these T-RFs generally decreased in abundance in the mice with DSS-induced colits.

T-RFs 90-94 can represent members belonging to *Bacteroidales* (other than *Parabacteroides*). These T-RFs were commonly found in both murine models and human samples and usually were one or the main driving forces for the gut microbiota clustering. As seen in the included studies in this thesis, members of *Bacteroidales* were active responders of various treatments.

T-RFs 117-119 can represent taxa in the genus *Allobaculum* (belonging to family *Erysipelotrichaceae*). These T-RFs were generally found abundant in C57BL/6 mice (in both LFD and HFD, **study A**). However, they were not detected in rats (**study D** and **G**, **paper I** and **II**) or BALB/cJ mice (fed standard rodent chow, **study H**) or in human samples (paper **III**, paper **IV**). The taxa represented by these T-RFs could be host specific or influenced by environmental factors such as different laboratory animal suppliers. The growth of these bacteria were seen to be favored by EPS (**study C**) but suppressed by green tea supplementation to a HFD (**study E** and **F**). HFD or western diet have been reported to shift the microbiota composition towards reduced *Bacteroidetes* but increased *Fimicutes*, which were mainly due to the proliferation of class *Erysipilotrichi* or at family level *Erysipelotrichaceae* [33, 36, 37, 107]. However, in **study A**, these bacteria were enriched in LFD fed mice (average abundance 10%) in comparison to the HFD fed ones (average abundance 3%). Similar observeation was reported by Ravuusin et al. [108], where low fat feeding (10% fat) enriched *Allobaculum* in comparison to the high fat feeding (60% fat). Species of *Allobaculum* have been reported to be mice strain specific [109]. The type species of *Allobaculum* has been shown to be able to produce butyrate [110] and it could be that EPS were utilized as extra carbon substrate that favored the growth of these bacteria. Green tea is known to have antimicrobial activities which may inhibit the growth of these bacteria.

T-RF 146 was identified as *Turicibacter* in **paper IV**. It was detected in the ileal pouch mucosal samples of some former UC patients one month and/or one year after surgery.

T-RF 217-221 were identified as taxa of *Lachnospiraceae* and these T-RFs were found occasionally in the murine models and in the human samples. T-RF 220 was found to be highly enriched in pectin fed rats in **study D**. On the other hand T-RF 221 was absent in the pectin group but were found in guar gum fed group. One base difference indicates that the taxa were closely related; however, they are favored or suppressed because of the different nature of the two fibers. These T-RFs were found abundant in ileal pouch mucosal samples of former UC patients in **paper IV.**

T-RF 265 was identified as "*Akkermansia*-like" bacteria and commonly detected in both animal and human samples. *Akkermansia*-like bacteria were seen increasing with high fat diet and by oat bran (**study B**), EPS or EPS producing *P. parvulus 2.6* (**study C**) supplementations. In contrast, "*Akkermansia*-like" bacteria were suppressed by guar gum or pectin addition. These bacteria tolerated green tea supplementation well and were found abundant in all the groups that received different doses of green tea powder. Bilberry favored the growth of "*Akkermanisa*-like" bacteria in mice that underwent ischemia-reperfusion (none of the mice in I/R control or I/R-Sham had this T-RF). In L-NAME treated rats, *Akkermansia* tolerated bilberry and the phenolic mixtures well (**paper I**). Interestingly, T-RF 265 was not detected in **paper II**. T-RF 265 was detected only in two rats fed raspberry, but was absent in rats fed blackcurrant **in study G**. In human fecal samples, *Akkermansia* were not abundant in all the research persons (**paper III**). Inverse correlation was seen between *Akkermansia* load in the small intestine and the metabolic parameters known as risk factors for T2D in **study E**. *Akkermansia muciniphila* is a mucin degrader [111] and found as an abundant resident in the human intestine [112, 113]. Inconsistent findings regarding association between the load of *Akkermansia* and body weight have been reported. Lean or normal weight individuals tend to have more *Akkermansia* in the fecal microbiota than overweight or obese ones [114, 115]. However, the prevalence of *Akkermansia* was lower in the stool samples taken from infants of normal weight mothers [116]. In addition, the prevalence of *Akkermansia* was found to be higher in the breast milk of overweight mothers [117]. The amount of *Akkermansia* was seen increasing with age in both lean and diet induced obese mice [118], and the enrichment of *Akkermansia* was found to be mice-strain specific [99]. Even though some promising health promoting effects of *Akkermansia* were suggested [49, 119, 120] , a recent study showed that the presence of *Akkermansia* worsened inflammatory state of a murine pathogen-infected mice which harbors simplified human gut microbiota [121]. The increased amount of *Akkermansia* in colonic mucosa of the mice with the DSS-induced colitis in **study I** is also suggesting that *Akkermansia* may work as an opportunistic pathogen in diseased states.

T-RF 275-277 were identified as *Faecalibacterium* and were commonly found in the human fecal samples in **paper III** and in the ileal pouch mucosal samples of former UC patients one year after surgery in **paper IV**.

T-RFs 291-294 can represent *Ruminococcaceae* and were found in both murine models and in human studies. These bacteria were increased by green tea (**study E** and **F**) or raspberry supplementation (**study G**) but not by bilberry (**study H, paper I** and **II**), and they were suppressed by oat bran (**study B**), EPS (**study C**), pectin or guar gum (**study D**). Unfortunately, the identity at lower taxonomic levels is not known for these T-RFs. Thus, the taxa that favor specific polyphenol rich food remains to be discovered in future studies. *Faecalibacterium prausnitzii* seems to be absent in mice and rats.

T-RF 497 was also commonly detected in both murine models and human studies. The bacterial group (or taxum) represented by this T-RF was especially abundent in C57BL/6J mice fed HFD with or without green tea+HEAL19 supplementation. Bilberry alone or together with probiotic strains also favored the growth of this taxum. Identification of this T-RF was difficult within the experiment setting used in this thesis. Primer 519R was used for sequencing which means due to the poor quality sequences in the first 30-50 bases, the length for theoretical identification of this T-RF was limited to shorter than 497. Even though a *Desulfovibrio* sp. were found to be able to generate this T-RF, an unidentified bacterial taxum could be represented by this T-RF.

T-RF 550 was identified as *Streptococcus* and found abudent in human oral microbiota in **paper III** and in the ileal pouch mucosal sample of one former UC patient one year after surgery in **paper IV**.

The T-RFs discussed above and some other T-RFs of interest in certain studies are summerized in table 2.

Effects of food supplements containing probiotics on the gut microbiota.

Probiotics were used in **study C, study E, study F, study G, study H, paper I, paper II** and **paper III**. Significant increase in the microbiota diversity achieved by probiotics alone was only seen in the **study F**. This is probably dependant on the long treatment period (22 weeks), but it may also give an indication on the stability of the gut microbiota. The observed increase in diversity was mainly due to the increase of the rare taxa. In **study E**, although not to the overall diversity, HEAL19 alone significantly increased richness (measured by the number of detected T-RFs) in the small intestinal mucosa associated microbiota. These findings points that HEAL19

promoted symbiosis in the intestine. Unlike the **study F**, no significant difference was seen in the cecal microbiota in **study E**. This could be explained by the difference in sampling. Cecal tissue samples were used in **study F** whereas cecal content was used in **study E**. The probiotic effect on the diversity of the densly populated luminal microbiota (more diverse system) could be more difficult to observe than in the mucosa attached microbiota (less diverse system) where strong binding capacity of the bacteria was required hence less densely populated.

In **study C**, *P. parvulus* 2.6 did not affect overall diversity but decreased bacterial richness. One of the supportive evidence could be the antagonized *Enterobacteriaceae*.

In **paper III**, HEAL19 alone or combined with bilberry did not exert any obvious effect on the oral and fecal microbiota. The robustness and the stability of the microbiota were underlined, at least for the fecal one. This has also been observed by others [122-125].

The effect of the combination of probiotics and berries on the microbiota was mainly driven by the berries. Unlike berries, synbiotic effect (e.g. promoted growth of *Lactobacillus* and attenuated HFD-induced inflammation) was achieved when combined HEAL19 with green tea. Dose effect of green tea was also observed when combining with HEAL19, such as the highest increase in bacterial diversity was seen when HEAL19 was combined with lower doses of green tea. To find the best berry partner for specific probiotic strains, a dose-dependent study might be a good starting point.

Table 2. Summary of T-RFs of interest detected in this thesis. Sizes of T-RFs were from *Msp*I digestion.

T-RFs	Diet Supplement/Treatment	Increase/Decrease	Putative Identification/Comments
80-88	DSS-colitis	Decrease	*Rikenellaceae and/or Parabacteroides*
88	Guar gum	Decrease	
88	Pectin	Decrease	
88	Bilberry	Increase	
88	Green tea	Increase	
84 and 87	Guar gum	Increase	
88	High fat	Tolerate	
90-93	Acitive responders to different diet treatments		Members of *Bacteroidales* e.g. *Bacteroides prevotella Barnesiella Rikenellaceae*
117-119	Green tea	Decrease	*Allobaculum* (family *Erysipelotrichaceae*) (common in C57BL/6 mice)
	EPS produced by *Pediococcus parvulus* 2.6	Increase	
	Low fat diet	Increase	
146	ileal pouch mucosa		*Turicibacter*
217-221			*Lachnospiraceae*
220	Pectin	Increase	
221	Guar gum	Tolerate	
217- 219	Ileal pouch mucosa		
265	High fat diet	Increase	*Akkermansia*
	Oat bran	Increase	
	EPS or *P. parvulus* 2.6	Increase	
	Guar gum	Decrease	
	Pectin	Decrease	
	Green tea	Tolerate	
	Bilberry (Ischemia-referfusion Balb/cj mice)	Increase	
	Bilberry or phenolic mixture (L-NAME)	Tolerate	

265	Raspberry/blackcurrant	Low prevalence	*Akkermansia*
	Human feces	Individual specific	
	Ileal pouch mucosa	Low prevalence	
275-277	Ileal pouch mucosa	common	*Faecalibacterium*
	Human feces	common	
289	Ileal pouch mucosa	Individual specific	*Megamonas*
291-294	Green tea	Increase	*Ruminococcaceae*
	Raspberry	Increase	
	Bilberry	Tolerate	
	Oat bran	Decrease	
	EPS	Decrease	
	Pectin	Decrease	
	Guar gum	Decrease	
488-491			*Enterobacteriaceae/Haemophilus* (family *Pasteurellaceae*)/*Sutterella* (family *Sutterellaceae*)
491	Human mouth	Abundent	*not identified*
497	High fat, green tea+HEAL19, bilberry+probiotics		*Desulfovibrio sp.*
515	Ileal pouch mucosa	Abundent	*Clostridium*
550	Human mouth	Abundent	*Streptococcus*
	Ileal pouch mucosa	Individual specific	

Conclusions and future perspectives

The trip to rediscover the bacteria residing along the GI tract is starting. Research on the gut microbiota and attempts to modulate the gut microbiota to gain health benefits are ongoing! We hope that the exploring of the microbiota of the GI tract done in this thesis can provide some more evidence for the further research and the designing of new functional food.

Gut microbiota of the two substrains of C57BL/6 mice responded differently to the same diet. The animals were purchased from different lab animal suppliers. Host genetics and/or environmental factors could shape microbiota differently. Difference in the gut microbiota structure may contribute to the different outcome of dietary interventions.

High fat diet can alter the gut microbiota dramatically. In C57BL/6 mice, we observed that "*Allobaculum*-like" (family *Erysipelotrichaceae*, phylum *Firmicutes*) bacteria were enriched in low fat (10% fat) fed mice compared to high fat (60%) fed mice. The growth of these bacteria was seen to be favored by EPS but suppressed by green tea. These bacteria were not detected in BALB/cJ mice and SD and Wistar rats used in this thesis.

Bacteroidales (Phylum *Bacteroidetes*) members were the most active responders to the diet treatments. *Rikenellaceae* and/or *Parabacteroides* were proliferated by bilberry and green tea but some of the members were suppressed by pectin and guar gum whereas some were favored by guar gum. In human fecal microbiota, *Bacteroides* and unclassified *Rikenellaceae* were found to be the most fluctuating bacteria. In the ileal pouch mucosal microbiota of former UC patients, *Bacteroides* was one of the most dominant genera.

Pectin boosted growth of bacterial members from *Lachnospiraceae* (phylum *Firmicutes*) and an unidentified bacterial group represented by T-RF 312. Pectin supplementation to the HFD led to the reduction in weight gain. It would be interesting to identify the bacteria at lower taxonomic level and to see if they can be expected to take part in the energy metabolism. In the ileal pouch mucosal microbiota of former UC patients one year after surgery, *Lachnospiraceae* was one of the most dominant members.

Ruminococcaceae (phylum *Firmicutes*) was increased by green tea or raspberry supplementation but not bilberry and suppressed by oat bran, EPS, pectin or guar gum. The bacterial composition that favors specific polyphenol rich food remains to be discovered in future studies.

Desulfovibrioceae (phylum *Proteobacteria*) and unidentified bacterial group represented by T-RF 497 were highly abundent in C57BL/6J mice fed HFD with or without green tea+HEAL19 supplementation. Bilberry alone or together with probiotic strains also favored the growth of the bacteria represented by this T-RF.

"*Akkermansia*-like" bacteria were seen increasing with high fat diet and by oat bran, EPS or EPS producing *P. parvulus 2.6* supplementations. However, they were suppressed by guar gum or pectin addition. These bacteria well tolerated green tea supplementation and were found abundant in all the groups that received different doses of green tea powder. Bilberry but not raspberry nor blackcurrant favored the growth of *Akkermanisa*-like bacteria in different studies. In human fecal samples, we found that *Akkermansia* are not abundant in all research persons. In mice with DSS-induced colitis, *Akkermansia* was increased compared to healthy controls.

Probiotic interventions with *L. plantarum* HEAL19 induced a significant increase in overall diversity after long time treatment, and the influences on the bacterial composition can be observed with shorter time in animal models. Green tea was found as a good partner to *L. plantarum* HEAL19, whereas combination to different berries (bilberry, raspberry, and blackcurrant) did not achieve optimum symbiotic effects.

In controlled animal studies, the influence of the dietary interventions on the gut microbiota composition was usually visible. Using the T-RFLP method we could observe the responses from dominant bacterial groups. With the help of deep sequencing methods, response from rare species and at lower taxonomic level could be detected. One can question the changes in the dominant or the rare species, which are more physiologically relevant? To answer this question, first step for the future study can be made to identify the active responders of *Bacteroidales* and find their roles.

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Paper I

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Research Article

Intake of Blueberry Fermented by *Lactobacillus plantarum* Affects the Gut Microbiota of L-NAME Treated Rats

Jie Xu,[1] Irini Lazou Ahrén,[2] Olena Prykhodko,[3] Crister Olsson,[1] Siv Ahrné,[1] and Göran Molin[1]

[1] *Department of Food Technology, Engineering and Nutrition, Lund University, 222 41 Lund, Sweden*
[2] *Probi AB, 223 70 Lund, Sweden*
[3] *Department of Biology, Lund University, 223 62 Lund, Sweden*

Correspondence should be addressed to Jie Xu; jie.xu@appliednutrition.lth.se

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Prebiotics, probiotics, or synbiotics can be used as means to regulate the microbiota to exert preventative or beneficial effects to the host. However, not much is known about the effect of the gut microbiota on hypertension which is a major risk factor of cardiovascular disease and also a symptom of the metabolic syndrome. The N^G-nitro-L-arginine methyl ester (L-NAME) induced hypertensive rats were used in order to test the effect of a synbiotic dietary supplement of *Lactobacillus plantarum* HEAL19 either together with fermented blueberry or with three phenolic compounds synthesized during fermentation. The experimental diets did not lower the blood pressure after 4 weeks. However, the fermented blueberries together with live *L. plantarum* showed protective effect on liver cells indicated by suppressed increase of serum alanine aminotransferase (ALAT) levels. The diversity of the caecal microbiota was neither affected by L-NAME nor the experimental diets. However, inhibition of the nitric oxide synthesis by L-NAME exerted a selection pressure that led to a shift in the bacterial composition. The mixture of fermented blueberries with the bacterial strain altered the caecal microbiota in different direction compared to L-NAME, while the three phenolic compounds together with the bacteria eliminated the selection pressure from the L-NAME.

1. Introduction

In recent years, the view of the gut microbiota seen as a metabolic organ has prompted intensive studies on the link between the microbiota and the host health. The plausible roles of the altered microbiota in the development of obesity and type 2 diabetes have been discussed [1, 2]. However, few studies have looked at the relationship between microbiota and hypertension, the latter being an important symptom of the metabolic syndrome and a major risk factor of cardiovascular disease.

Hypertension characterized as elevated systolic and/or diastolic blood pressure (SBP ≥ 140 mmHg, DBP ≥ 90 mmHg, resp.) is usually treated with antihypertensive agents, but life style modification has also been recommended for both prevention and treatment [3, 4]. One approach to the dietary intervention is to use probiotics together with dietary fibers having prebiotic potential. However the yielding results have so far been inconsistent in improving the hypertensive conditions in both animal models and human trials. In a spontaneously hypertensive rat (SHR) model, administration of milk fermented with *Lactobacillus paracasei* subsp. *paracasei* NTU 101 or *Lactobacillus plantarum* NTU 102 either as a single dose or for 8 weeks, both, significantly decreased SBP and DBP. The authors postulated that the underlying mechanism of the antihypertensive effect could be the result from two substances produced by the two bacteria strains, that is, angiotensin I-converting enzyme inhibitor (ACEI) which is supposed to block the conversion of angiotensin I to angiotensin II and a neurotransmitter γ-aminobutyric acid (GABA) [5]. In another study, supplementary tablets made from *Lactobacillus helveticus* CM4 in fermented milk powder were given to people with high-normal blood pressure (SBP = 130–139 mmHg, DBP = 85–89 mmHg) and mild hypertension (SBP = 140–159 mmHg, DBP = 90–99 mmHg) for 4 weeks. A significant decrease in DBP by 5 mmHg but

not in SBP was seen in the high-normal blood pressure group, while SBP was significantly decreased by 11.2 mmHg and DBP decreased by 6.5 mmHg ($P = 0.055$) in the mild hypertension group [6]. In contrast, no antihypertensive effect was seen when *Lactobacillus helveticus* Cardio04 in fermented milk was given to human subjects for 8 weeks [7].

Dietary flavonoid intake has been shown to have multiple health beneficial effects [8, 9]. Flavonoids, a major group of polyphenols, encompass structurally diverse subclasses which are naturally found in fruits, vegetables, berries, and dark chocolates as well as beverages such as tea and wine. Recent meta-analysis studies confirmed that some of these phenolic compounds such as cocoa flavonols and soy isoflavones were able to reduce blood pressure effectively [10, 11]. Consumption of blueberries (*Vaccinium* spp.), a good source of dietary flavonoids, has also been shown to have beneficial effects on hypertension. Rats fed with a blueberry enriched diet for 6 or 12 weeks showed decreased blood pressure [12]. An antihypertensive effect was also seen in another rat model when the animals were fed blueberry extract for 4 or 6 weeks [13]. Humans with metabolic syndrome who consumed a beverage containing 50 g of freeze dried blueberries for 8 weeks showed a significant decrease in both SBP and DBP [14]. The positive effects were mostly attributed to the antioxidant capacities of anthocyanins. In addition to polyphenols, blueberry also contains essential nutritional components such as vitamin C, folic acid, and minerals, as well as dietary fibers [15].

The antihypertensive effect of using probiotics and blueberries can vary depending on the bacterial strain and blueberry species used and its growth conditions. In the present study we hypothesize that blueberry (*Vaccinium myrtillus*; bilberry in American English) fermented by *Lactobacillus plantarum* HEAL19 (DSM 15313), a tannase producing strain, would exert synbiotic effect on improving the hypertension by modulating the gut microbiota. To test the hypothesis and further elucidate whether the prebiotic effect is from the probiotic fermented whole blueberry or the phenolic compounds found after fermentation, L-NAME induced hypertensive rats were fed with either *L. plantarum* HEAL 19 together with the whole blueberries fermented by the same bacterial strain or with novel phenolic compounds found in the blueberries only after fermentation.

2. Materials and Methods

All experiments followed the national guidelines (SFS 1988:534 Swedish Animal Welfare Act, http://www.government.se/content/1/c6/09/03/10/f07ee736.pdf/) for the care and use of animals and were approved by the Malmö/Lund regional ethical committee for laboratory animals (permission number M 83-10).

2.1. Animals. Adult male rats (*Rattus norvegicus*) of the Sprague Dawley strain (Mol: SPRD Han, Taconic M & B, Denmark) weighing 200–250 grams (12-13 weeks old) were used in the experiment. The rats were kept in the animal facility at the Department of Biology, Lund University, under pathogen free conditions (20 ± 1°C, 50 ± 10 RH%, 12 : 12 hrs light-dark cycle) in polycarbonate cages on aspen wood bedding material with free access to the water and rodent laboratory chow (product number R34; Lantmännen, Stockholm, Sweden) placed on the lid of cages. Prior to the study, all animals were randomly divided into experimental groups on arrival day with 9 animals per group and kept by three rats per cage during the whole study. Before any experimental procedures, the animals were allowed to acclimatize to their new environment for 7–10 days. At the end of the experiment, the rats were anesthetized with a 3 mL/kg of subcutaneous injection of Hypnorm (fentanyl citrate 0.15 mg/mL; fluanisone 10 mg/mL, Janssen, Oxford, UK) and Dormicum (midazolam, 5 mg/mL, Roche AB, F. Hoffmann-La Roche Ltd. Basel, Switzerland) diluted each in an equal amount of water prior to their mixture in a final ratio of 1 : 1 : 2, respectively.

2.2. L-NAME Model. The hypertensive state in the rats was induced by adding the nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) in the drinking water at 400 mg/L. The rats received approximately 40 mg/kg/day of L-NAME for 4 weeks.

2.3. Diets/Treatment. Rats were fed standard chow with or without the addition of one of the two study products tested. One study product consisted of blueberries (*Vaccinum myrtillus*) that had been fermented by *Lactobacillus plantarum* HEAL19 (DSM 15313), which is a bacterial strain with a strong tannase activity that can efficiently break down tannins found in blueberries into smaller phenolic acid compounds. The fermented blueberries were freeze dried and milled into a powder. Such powder was given to the rats at 2 g/rat/day (product FBL) mixed with standard chow. The second study product (product PML) consisted of a mixture of three phenolic acids that were absent in the nonfermented blueberries but were detected in the fermented blueberry product. The three phenolic acids were added in the amount that corresponded to their concentration in the daily dosage of product FBL. The phenolic acid mixture consisted of 518 μg of hydroxylactic acid (Sigma, H3253), 2540 μg of phenyllactic acid (Sigma, 113069), and 278 μg of 3,4-dihydroxyphenylpropionic acid (Sigma, 102601). All rats receiving blueberry powder or the phenolic acid mixture were also given 10^9 cfu/animal/day of *Lactobacillus plantarum* HEAL19 (HEAL19; DSM 15313) by mixing the power with standard chow. The bacterial dose was chosen based on experience from other animal studies with lactobacilli and to reach the amount that could lead to better chance of having positive effect but not too high to make it nonrealistic when converted for possible application to humans. Small boluses were formed by premixing the 15 g of the rodent standard feed in powdered form with 3 mL of water and corresponding product that was recalculated for each rat. The boluses were placed in the "food compartment" on the cage instead of pellets chow for overnight, when rats are most active with eating. Next morning, the pelleted chow was returned, while the rest of the uneaten boluses was removed

and recorded. The control group obtained similarly prepared boluses without tested products. Administration of the study products started on the same day as the administration of L-NAME in the drinking water. The consumed amounts of drinking water per cage were recorded every day before administration of fresh water and feed. The following groups of animals were included in the study: (i) control animals not treated with L-NAME receiving standard chow (Ctrl), (ii) L-NAME treated rats receiving standard chow (LN), (iii) L-NAME treated rats receiving blueberry fermented with *L. plantarum* HEAL19 and live HEAL19 (LN + FBL), and (iv) L-NAME treated rats receiving the mixture of three phenolic compounds and live HEAL19 (LN + PML). One animal in the LN + FBL group lost weight and was excluded from data analysis.

2.4. Blood-Pressure Measurement. The blood pressure was measured by using the tail-cuff method (CODA, Kent scientific corporations, Torrington, CT, USA) following the manufacturer's instructions. To ensure good circulation of the blood in tail, the animals were kept under heating lamp for the several minutes prior to blood pressure measurement. Then, in quite conditions, the animals were placed into the nose-cone holders on the heating table accompanied the CODA 2 system. After 10 minutes of adaptation to the holders, the cuffs were applied on the tail and 1 run of acclimatization cycle was performed. Totally, 2 sets with 4-5 cycles were performed and result was calculated as a mean per each blood pressure measurement. If animal showed increased heart rate, indicating stress, the blood pressure measurement was repeated later but at the same day. Blood pressure levels were measured at baseline and after 2 and 4 weeks of intervention/treatment. Both systolic (SBP) and diastolic blood pressure (DBP) were measured. Prior to the beginning of study, the animals were trained to the blood pressure measurements to minimize the effect of stress during experimental procedure.

2.5. Body Weights. The rats were weighed at baseline and after 2 and 4 weeks of treatment. They were weighed just before having their blood pressure measured.

2.6. Blood Sample Analysis. At the end of the experiment, the anesthetized rats were opened and the blood was taken by cardiac puncture into vacutainers for serum. After centrifugations for 10 min at 3000 ×g at 4°C, the serum was collected and stored at −70°C until analysis. Serum alanine aminotransferase (ALAT) was analyzed at the Department of Clinical Chemistry (SUS, Malmö, Sweden) using a COBAS 6000 analyzer (Roche) according to routine methods.

2.7. Caecum Samples. After the opening of the abdomen, the caecal content for microbiological analyses was collected into sterile 2 mL cryovials, immediately placed on dry ice, and kept frozen at −70°C until analyses.

2.8. Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis. Total DNA was extracted from the caecal content with EZ1 DNA tissue kit (Qiagen AB, Sollentuna, Sweden) on a BioRobot EZ1 workstation (Qiagen). Briefly, approximately 50 mg of the caecal content was suspended in 500 μL of sterile 1x PBS buffer (Oxoid, Basingstoke, UK) and vortexed for 1 min. A sterile 1 μL loop was used to disintegrate big clumps if seen and then 30 min of bead beating step was done on an Eppendorf Mixer (Model 5432, Eppendorf, Hamburg, Germany). Followed by this mechanical lysis, the sample was incubated at room temperature for 10 min, vortexed for 1 min, and centrifuged at 600 ×g for 30 seconds. Then 200 μL of the supernatant was transferred to a sterile sample tube (Qiagen) and total DNA was extracted according to the manufacturer's instructions. The 16S rRNA gene was amplified by using a universal primer pair (Thermo Fisher Scientific, Ulm, Germany) ENV1 (5′-AGAGTTTGATIITGGCTCAG-3′) and ENV2 (5′-CGGITACCTTGTTACGACTT-3′). The 5′ end of ENV1 was fluorescently labelled with FAM dye. The PCR reaction was set up in a total volume of 25 μL consisting of 0.4 μM of FAM-ENV1 primer and 0.2 μM of primer ENV2, 2.5 μL of 10x PCR reaction buffer (500 mM Tris-HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, pH 8.3), 0.2 mM of each deoxyribonucleotide triphosphate, 1.25 U of FastStart Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), and 10–20 ng of template DNA. The PCR was performed under the following condition: 95°C for 3 min, 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 50°C for 45 s, and 72°C for 2 min. Finally, an additional extension at 72°C for 7 min was done. To decrease the PCR bias, triplicate PCR reactions were prepared for each sample and a negative control was included in every run. The correct amplicon size was checked by agarose gel electrophoresis after each PCR. The resulting PCR products from each sample were pooled together and purified with MinElute PCR purification kit (Qiagen). Then, the DNA concentration was measured by NanoDrop ND-1000 (Saveen Werner, Limhamn, Sweden) and 200 ng of the purified DNA was digested with the endonuclease *Msp*I (Fermentas Life Science, Burlington, Canada) in a total volume of 10 μL according to the manufacturer's instruction. The digested amplicons were analysed on an ABI 3130xl Genetic analyzer (Applied Biosystems, Foster city, CA, USA) with internal size standard GeneScan LIZ 600 (range 20–600 bases, Applied Biosystems) at DNA-lab (SUS, Malmö, Sweden). The resulting T-RFLP data was analyzed using the GeneMapper software version 4.0 (Applied Biosystems) with local southern algorithm. T-RFs were resolved between 40 and 580 bases considering four internal standards were required for accurate sizing of an unknown T-RF. The relative area percentage was calculated for each T-RF which then was used for diversity calculation and principal component analysis (PCA).

2.9. SYBR Green Quantitative PCR (qPCR). Recombinant plasmid standards were constructed by cloning the corresponding 16S rRNA gene fragments specific for the bacterial species into the pGEM-T Vector Systems (Promega, Madison, USA). DNA extracted from bacterial pure cultures was used

Table 1: Primers and annealing conditions used in qPCR for bacterial quantification.

Name	Sequence (5′-3′)	Target group	Amplicon size (bp)	Annealing temperature (°C)	Reference
Lact-16S-F	GGAATCTTCCACAATGGACG	*Lactobacillus*	217	56	[16]
Lact-16S-R	CGCTTTACGCCCAATAAATCCGG				
Eco1457-F	CATTGACGTTACCCGCAGAAGAAGC	*Enterobacteriaceae*	195	60	[17]
Eco1652-R	CTCTACGAGACTCAAGCTTGC				
g-Bfra-F	ATAGCCTTTCGAAAGRAAGAT	*Bacteroides fragilis* group	495	50	[18]
g-Bfra-R	CCAGTATCAACTGCAATTTTA				
AM1-F	CAGCACGTGAAGGTGGGGAC	*Akkermansia*	327	60	[19]
AM2-R	CCTTGCGGTTGGCTTCAGAT				
Tot-F	GCAGGCCTAACACATGCAAGTC	Total bacteria	292	60	[20]
Tot-R	CTGCTGCCTCCCGTAGGAGT				

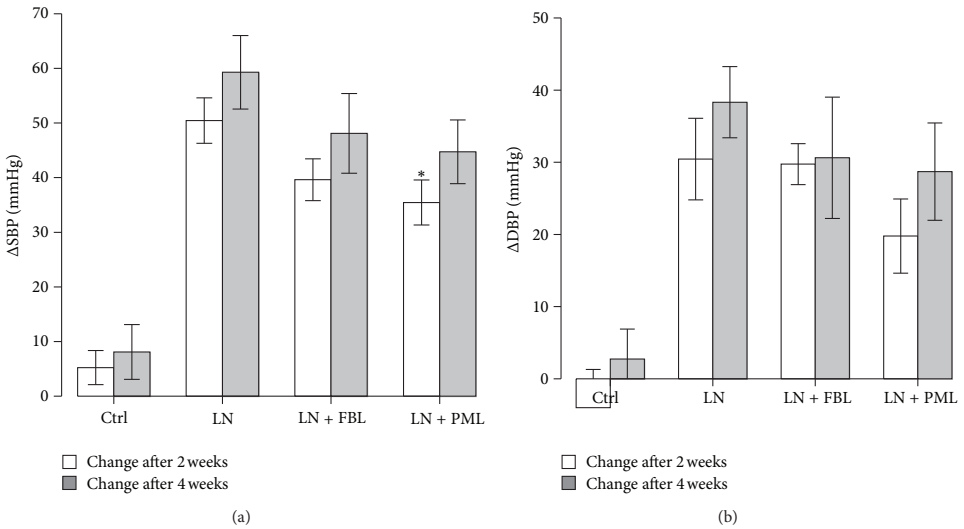

Figure 1: Blood pressure changes after 2 and 4 weeks treatment. (a) Changes in SBP. (b) Changes in DBP. Ctrl: control ($n = 9$); LN: group receiving standard chow and L-NAME in drinking water ($n = 9$); LN + FBL: group receiving L-NAME in drinking water and fermented bluberry + *L. plantarum* HEAL19 added to feed ($n = 8$); LN + PML: group receiving L-NAME in drinking water and three phenolic acid mixture + *L. plantarum* HEAL19 added to feed ($n = 9$). Both SBP and DBP significantly increased in L-NAME treated groups after 2 and 4 weeks when compared to the Ctrl. Data are means ± SE. $^*P < 0.05$ compared to LN.

as follows: *L. plantarum* CCUG 35035 (Culture collection, University of Gothenburg, Sweden) was used to amply the specific region for *Lactobacillus* and total bacteria: *Escherichia coli* CCUG 29300 for *Enterobacteriacea*e, and *Bacteroides fragilis* ATCC 25285 for *B. fragilis* group, and a *Akkermansia* clone obtained from a mouse caecum was used for targeting *Akkermansia muciniphila* specific region on the 16S rRNA gene. The bacterial groups were amplified using QuantiTect SYBR Green PCR kit (Qiagen) in a real-time PCR cycler Rotor-Gene Q (Qiagen). The primers and annealing temperatures used in the study are listed in Table 1. The PCR profile was set as follows: activation at 95°C for 15 min followed by 40 cycles of (1) 95°C for 15 sec, (2) annealing at 50–60°C for 30–60 sec, and (3) extension at 72°C for 30 or 60 sec. Melt curve analysis was performed for each run to check the specificity of the primers. The PCR reaction was prepared in 20 μL consisting 10 μL of 2x QuantiTect SYBR Green PCR Master mix, 0.5 μM of each primer, and 2 μL of template DNA. If necessary, template DNA was diluted 10–100-fold prior to qPCR. Triplicate reactions were performed for each sample, standard, and negative controls.

2.10. Statistics. The blood pressure and body weight data were normally distributed and analysed with one-way ANOVA and a Tukey HSD post hoc test for pairwise comparison if necessary using package "stats" in the R (version 2.15.1) program. The values are presented as the mean ± SE. Values of $P < 0.05$ were considered statistically significant.

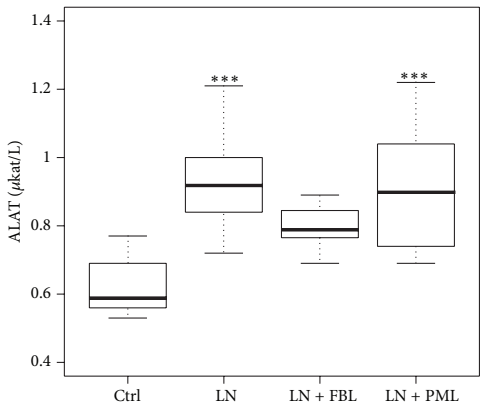

Figure 2: Serum ALAT enzyme. Ctrl: control ($n = 9$); LN: group receiving standard chow and L-NAME in drinking water ($n = 9$); LN + FBL: group receiving L-NAME in drinking water and fermented bluberry + *L. plantarum* HEAL19 added to feed ($n = 8$); LN + PML: group receiving L-NAME and three phenolic acid mixture + *L. plantarum* HEAL19 ($n = 9$). $^{***}P < 0.001$ compared to Ctrl.

Table 2: Caecal microbiota diversity indices.

	Shannon's diversity indices	
	Median	25–75%
Ctrl	2.90	2.82–2.99
LN	2.96	2.85–2.98
LN + FBL	2.72	2.55–2.88
LN + PML	2.97	2.82–2.99

Ctrl: control ($n = 9$); LN: group receiving standard chow and L-NAME in drinking water ($n = 9$); LN + FBL: group receiving L-NAME in drinking water and fermented bluberry + *L. plantarum* HEAL19 added to feed ($n = 8$); LN + PML: group receiving L-NAME and three phenolic acid mixture + *L. plantarum* HEAL19 ($n = 9$). Data are expressed as median values and 25–75 percentiles.

The ALAT enzyme, Shannon's diversity index [21], and qPCR data were not normally distributed and thus evaluated with nonparametric test Kruskal-Wallis test and Nemenyi-Damico-Wolfe-Dunn test (NDWD) for pairwise comparisons using package "coin" in the R program. Values of $P < 0.05$ were considered statistically significant.

T-RFLP data was analyzed with principal component analysis (PCA) with SIMCA-P software (version 12.0.1.0; Umetrics, Umeå, Sweden).

3. Results

3.1. Normal Growth of Animals in All Groups. The experimental diets did not affect the normal growth of the animals. There was no significant difference in body weight gain after 4 weeks between groups (76.1 ± 5.0 g, 85.1 ± 4.4 g, 83.4 ± 5.6 g, 83.3 ± 8.5 g for Ctrl, LN, LN + FBL, and LN + PML, resp.).

3.2. Experimental Diets Had No Effect on Blood Pressure. The L-NAME treatment significantly elevated both SBP ($P < 0.001$) and DBP ($P < 0.001$) after 2 weeks. The significant increase in BP remained after 4 weeks ($P < 0.001$ for SBP and $P < 0.01$ for DBP). There was a reduction in SBP after 2 weeks in LN + PML group ($P = 0.04$) compared to LN. However, the experimental diets had no effect either on SBP or DBP after 4-week treatment (Figure 1).

3.3. LN + FBL Did Not Increase Liver Enzyme ALAT. The serum ALAT levels were significantly increased in LN ($P < 0.001$) and LN + PML ($P < 0.001$) groups compared to Ctrl (Figure 2). However, no statistically significant increase was detected in LN + FBL ($P = 0.06$).

3.4. No Change in Microbial Diversity after Inducing Hypertension. Shannon's diversity index was used to compare the microbial diversities. Neither the L-NAME treatment nor the experimental diets did change the caecal microbial diversity (Table 2).

3.5. Certain Bacterial Population Altered after Experimental Diet in Rats with Hypertension. qPCR was performed to see whether the induction of hypertension or the experimental diets would alter the bacterial composition in the caecum content (Table 3). Induction of hypertension by L-NAME did not significantly change any of the tested bacterial population. Even though not statistically significant, there was a trend of increase in *Enterobacteriaceae* population in LN group ($P = 0.064$). The supplementation of the FBL significantly decreased the *Lactobacillus* ($P = 0.023$) and *B. fragilis* group ($P = 0.016$) populations compared to LN but not to Ctrl. The addition of the PML showed a trend of increase in *Akkermansia* population compared to LN group ($P = 0.051$). The expected increase in *Lactobacillus* was not seen either in LN + FBL or LN + PML group; this could be due to the unsuccessful colonization of the *L. plantarum* HEAL19.

3.6. Microbiota Compositional Change after Hypertension Induction. The PCA analysis was applied on the T-RFLP data to illustrate the variation of the caecal microbiota between the groups. The data matrix composed of relative area of the T-RFs was not scaled but centered and the first two principal components (PCs) were calculated. PC1 explained 31.1% of the variance and PC2 explained 17.7%. PCA loadings biplot (Figure 3) showed the correlation between T-RFs representing different bacteria groups and also the similarity of the microbiota between the individual animals based on the T-RF distribution. LN + FBL group was separated from other groups mostly by PC2 and showed large individual variation scattered in the first and second quadrants. In addition, bacteria groups represented by T-RF88 and T-RF533 were most abundant in LN + FBL group. Animals from LN group were found mostly in the third quadrant except for one rat was found in the fourth quadrant, suggesting that they were sharing more homogeneous microbiota. LN group harbored high abundance of T-RF93 and T-RF217 but suppressed bacteria group represented by T-RF303. Animals

Table 3: SYBR Green qPCR of bacterial 16S rDNA in rat caecal content.

	Ctrl		LN		LN + FBL		LN + PML	
	Median*	25–75%	Median	25–75%	Median	25–75%	Median	25–75%
Lactobacillus	8.35^{ab}	7.93–8.77	8.54^{b}	8.50–8.86	7.84^{a}	7.67–8.16	8.96^{b}	8.71–9.07
Enterobacteriaceae	7.64	7.39–7.77	8.07	7.83–8.14	7.14	6.64–7.76	7.64	7.43–7.95
Bacteroides fragilis group	11.26^{ab}	11.19–11.64	11.82^{b}	11.13–11.99	9.2^{a}	7.45–11.00	11.18^{ab}	10.86–11.86
Akkermansia	8.63	8.00–9.15	8.60	8.09–8.89	8.56	8.14–9.06	9.23	9.17–9.50
Total bacteria	10.17	10.05–10.42	10.03	9.61–10.60	9.97	9.72–10.18	10.18	9.79–10.23

Ctrl: control ($n = 9$); LN: group receiving standard chow and L-NAME in drinking water ($n = 9$); LN + FBL: group receiving L-NAME in drinking water and fermented blueberry + *L. plantarum* HEAL19 added to feed ($n = 8$); LN + PML: group receiving L-NAME and three phenolic acid mixture + *L. plantarum* HEAL19 ($n = 9$). Data are expressed as median values and 25–75 percentiles. Groups marked with superscript letters that do not share the same letter were significantly different ($P < 0.05$). *Data are expressed as median log copy/g and 25–75 percentiles.

from Ctrl and LN + PML groups were mostly located in the third and fourth quadrants, indicating that the LN + PML combination did not affect the caecal microbiota.

4. Discussion

None of the experimental diets had a blood pressure lowering effect, neither on SBP nor on DBP after 4 weeks. However, the experimental diet FBL which is the supplementation of *L. plantarum* HEAL19 with blueberries fermented by the same bacterial strain reduced the liver cell damage as observed by suppressed increase of serum ALAT level. ALAT, an enzyme mainly found in cytosolic side of liver cells, would leak into the blood stream if the liver cell is damaged. Elevated serum ALAT level has been reported to be associated with various liver diseases [22, 23]. L-NAME treatment significantly increased the ALAT level in this study. However, experimental diet FBL seemed to counteract the adverse effect of L-NAME on the ALAT increase, suggesting a possible protecting effect on hepatocytes. Consumption of blueberries has been reported to reduce hepatocyte injury, lipid peroxidation [24], and oxidative stress [25, 26]. Furthermore, fermentation of blueberries has been shown to increase the total polyphenols and antioxidant capacities [27, 28]. Thus, the fermentation of blueberries may have enhanced the scavenging of free radicals that may antagonize the development of liver injury.

Nitric oxide, a hydrophobic signaling molecule which is small enough to pass plasma membranes freely, is known to be involved in various physiological functions including antimicrobial activity [29, 30]. L-NAME that blocks the NO production has been shown to also decrease oxygen delivery to the gut [31]. Thus, it is plausible to assume that inhibition of the nitric oxide synthesis by L-NAME could have an effect on the oxygen tension in the microenvironment close to the intestinal mucosa, which might favor some components of the microbiota and disfavor others. In the present study, a shift was seen in the composition of the microbiota in the LN group by PCA analysis. L-NAME treatment has been reported to increase the viable count of *Enterobacteriaceae* in the gut of rats [32]. Although not statistically significant, a trend of increase in *Enterobacteriaceae* after L-NAME treatment was seen in this study but the supplementation of the FBL or PML seemed suppressed the growth of the *Enterobacteriaceae* (Table 3). This could be attributed to the antimicrobial effects from the experimental diets. In addition, L-NAME treatment also increased the total bacterial load in the caecum and colon in rats with liver injury [33]. However, we did not find significant differences in total bacteria load measured by qPCR in the present model which might be partly explained by the difference between culture-dependent and independent methods that were used.

L-NAME treatment has been reported to prevent the bacterial translocation by protecting the integrity of the GI tract in hemorrhagic shocked animals [34], while in healthy rats, administration of L-NAME increased the intestinal motility and the load of mucosa associated bacteria and the effects were suggested to be a consequence of a decreased mucus secretion [35]. In the present study, the amount of *Akkermansia* that are mucin degrading bacteria did not decrease after L-NAME treatment, which implies that mucus secretion has been unaffected in the present model, at least in the caecum. However, further studies on mucin gene expressions and quantification of the caecal mucin content are needed to confirm such a relationship.

The addition of the *L. plantarum* HEAL19 did not result in an expected increase of *Lactobacillus* population either in LN + FBL or LN + PML group. The T-RF568 that was putatively identified as the *L. plantarum* HEAL19 in previous work [36] has been only detected in one rat from the LN + FBL with low abundance around 0.5%. Thus it can be speculated that the survival rate of the bacterial strain was low. Antimicrobial activities of different blueberry species were observed to selectively inhibit growth of pathogens when tested as whole berry, berry fractions, extracts of phenolic compounds, and ethanol extract [37–39]. The significantly decreased population of *Lactobacillus* and *B. fragilis* group in the LN + FBL group as compared with LN group could be a result from such selection pressure of the blueberries. Despite the effects of the diets observed on the certain group of bacteria measured by qPCR, an overview of the caecal microbiota composition by PCA analysis revealed that large variations exist among the individuals upon the response to treatments. One contributing factor could be the different individual's original microbiota.

Interestingly, the phenolic compounds together with *L. plantarum* HEAL19 did not affect the composition of the microbiota as shown by PCA analysis (Figure 3). The

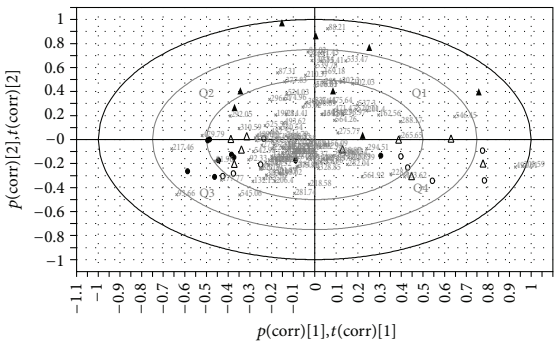

Figure 3: PCA loadings biplot. Open circle (Ctrl): control rats ($n = 9$); dots (LN): rats receiving standard chow and L-NAME in drinking water ($n = 9$); triangles (LN + FBL): rats receiving L-NAME in drinking water and fermented bluberry + *L. plantarum* HEAL19 added to feed ($n = 8$); open triangles (LN + PML): rats receiving L-NAME in drinking water and three phenolic acid mixture + *L. plantarum* HEAL19 added to feed ($n = 9$). The numeric numbers indicate the sizes in bases of the detected T-RFs representing different bacterial groups. Q1–Q4 denote 1st to 4th quadrants.

selection pressure from either L-NAME or phenolic compounds seemed to be eliminated. Phenyllactic acid (PLA) and hydroxyphenyllactic acid (HPLA), metabolites of phenylalanine and tyrosine, respectively, have previously been shown to have antibacterial and antifungal activities and can also be produced by *Lactobacillus plantarum* stains [40–42]. In the present study no such effect was observed on the caecal microbiota. It can be speculated that the compounds did not reach the effective concentration in the caecum. Another phenolic compound included in the experimental diet 3,4-dihydroxyhydrocinnamic acid (DHCA) is a metabolite of caffeic acid. A recent study has shown that DHCA enhanced the activity of endothelial nitric oxide synthase (eNOS) *in vitro* [43]. This provides a possible reason for the observation in PCA analysis that shows that the L-NAME effect on the microbiota was eliminated by the PML diet.

In conclusion, the main findings of this study are that (1) L-NAME alone conferred selective pressure on the caecal microbiota, (2) L-NAME with blueberries fermented with *L. plantarum* HEAL19 showed protective effect against liver cell damage and altered the caecal microbiota to different degrees in different individuals, and (3) the combination of the three phenolic compounds found in blueberries fermented with *L. plantarum* HEAL19 eliminated selection pressure on the caecal microbiota induced by L-NAME.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Paper II

Antihypertensive activity of blueberries fermented by *Lactobacillus plantarum* DSM 15313 and effects on gut microbiota in healthy rats

Irini Lazou Ahrén[1], Jie Xu*[2], Gunilla Önning[1], Crister Olsson[3], Siv Ahrné[2], Göran Molin[2]

[1]Probi AB, Lund, Sweden
[2]Laboratory of Food Hygiene, Department of Food Technology, Engineering and Nutrition, Lund University, Box 124, SE-221 00 Lund, Sweden
[3]Department of Biosystem and Technology, Swedish University of Agricultural Science, P.O. Box 103, 23053 Alnarp, Sweden

*Corresponding author. Email: Jie.Xu@appliednutrition.lth.se Phone: +46-46-222 98 16

Abstract

Purpose The aim of the present animal study was to examine the anti-hypertensive capacity of two probiotic products combining blueberries and the tannase producing probiotic bacteria *Lactobacillus plantarum* DSM 15313 and to clarify effects on gut microbiota in healthy rats.

Methods Male Sprague Dawley rats were treated with N^G-nitro-L-arginine methyl ester (L-NAME) in the drinking water (40mg/L) to induce a hypertensive state. Administration of the study product was initiated in parallel with L-NAME and lasted for 4 weeks. Two blueberry products differing in their phenolic acid content were tested and each rat received 2 g/day of the fermented blueberry powders. Six groups with 9 animals each were in total included in this study. Three of them received L-NAME in their water and three did not and in each setting there were rats that were used as controls and rats receiving either one of the blueberry products.

Results Intake of fermented blueberries significantly reduced the systolic and diastolic blood pressure of L-NAME treated rats after two weeks of intervention. After four weeks, there was a significant reduction of the blood pressure in non-L-NAME treated animals. The probiotic product with a higher content of phenolic acids reduced ALAT and increased plasma HDL. Furthermore, ingestion of the blueberry products resulted in changes of the gut microbiota in non-L-NAME treated rats.

Conclusions Blueberries fermented with the tannase producing bacteria *L. plantarum* DSM 15313 have anti-hypertensive properties and may reduce the risk for cardiovascular diseases.

Keywords

berries; hypertension; probiotics; microbiota; L-NAME

Introduction

High blood pressure or hypertension is the major risk factor for cardiovascular diseases (CVD) and is one of the risk factors used in the definition of the metabolic syndrome [1]. Other known risk factors for CVD are abnormal levels of blood lipids, insulin resistance, abdominal fat and systemic low-grade inflammation. Hypertension is defined as a systolic blood pressure ≥ 140 mmHg or a diastolic blood pressure ≥ 90 mmHg. According to the American Heart Association (www.americanheart.org), in the United States 74.5 million people or one in three adults have a high blood pressure. The costs for treating high blood pressure in the United States in 2010 were estimated to $76.6 billion in health care services, medications, and missed days of work [2].

Knowing and regularly measuring our blood pressure is important since the onset of hypertension is seldom associated with any symptoms. However, a non-treated high blood pressure can gradually damage vital organs such as the heart, the kidneys and blood vessels. Moreover, the earlier the treatment begins the better the clinical outcomes for the patient.

In recent years, many studies have focused on the anti-hypertensive or pre-hypertensive activity of food and food-substances as an alternative or a complement to pharmaceuticals. Dark chocolate [3], red wine polyphenols [4], beetroot [5] and lactotripeptides [6] are examples of foods and other non-pharmaceutical products that have been shown, either in animal or in clinical trials, to be able to reduce the blood pressure. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer health benefits on the host" [7]. The role of the gut microbiota on different diseases of the industrialized world is currently a hot topic for discussion and research [8-11]. It was commonly observed that a change in the host health status was accompanied by a shift in the gut microbiota. Thus, it is worth to investigate if we can gain health benefits by modulating the gut microbiota. The scientific interest on a possible effect of probiotics in reducing the risk for cardiovascular diseases is arising and there is a need for more studies within this field.

In the present study, we aimed at testing the anti-hypertensive activity of blueberries fermented by a tannase active, probiotic strain of *L. plantarum* (DSM 15313) in a rat model for hypertension. High blood pressure was induced by treating the rats with the nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME). The caecal microbiota was analysed in order to search for a link between reduced BP and change in the composition of the microbiota.

Materials and Methods

Animals. Adult male rats (*Rattus norvegicus*) of the Sprague-Dawley strain (Mol: SPRD Han; Taconic M & B, Denmark), 200-250 grams (12-13 weeks old) were used in the experiment. The rats were kept in the animal facility at the Dept. of Biology, Lund University under SPF conditions (20±1°C, 50±10 RH%, 12:12hrs light-dark cycle) in polycarbonate cages on aspen wood bedding material with free access to the water and rodent laboratory chow placed on the lid of cages. Before the experimental procedure the animals were randomized in groups and study sets, placed three rats per cage and allowed to adapt to the new environment for approximately 10 days. At the end of the experiment the rats were anesthetized with a 3 ml/kg of subcutaneous injection of Hypnorm (fentanyl citrate 0.15mg/ml; fluanisone 10mg/ml, Janssen, Oxford) and Dormicum (midazolam, 5 mg/ml, Roche AB, F.Hoffmann-La Roche Ltd Basel, Schweiz) diluted each in an equal amount of water prior to their mixture in a final ratio of 1:1:2, respectively.

Diets/treatment. Rats were fed standard chow (R36; Lantmännen, Sweden) with or without added freeze-dried fermented blueberry powder at 2 g/rat/day. The blueberries had been fermented over night after incubation with *Lactobacillus plantarum* DSM 15313 (=HEAL19). Two different fermented blueberry powders were tested and were described as product A and product B. They differed in their phenolic acid content, with product A having a higher concentration of certain phenolic acids (hydroxyphenyllactic acid,

3,4-dixydroxyphenyl-propionic acid and phenyllactic acid). The rats receiving blueberry powder were also given 10^9 cfu/day of *L. plantarum* DSM 15313 mixed with the blueberry powder and the standard chow. Administration of the blueberry powder started on the same day as the administration of L-NAME in the drinking water. The consumed amounts of drinking water and feed per cage were registered every day before administration of fresh water and feed. The following six groups with nine animals each were included in the study: i) animals not treated with L-NAME receiving standard chow (cont W), ii) animals not treated with L-NAME receiving product A and bacteria (W+A), iii) animals not treated with L-NAME receiving product B and bacteria (W+B), iv) L-NAME treated rats receiving standard chow (cont LN), v) L-NAME treated rats receiving product A and bacteria (LN+A) and vi) L-NAME treated rats receiving product B and bacteria (LN+B). The animals were randomly allocated to each group.

Sample size. The sample size in this study was based on the results obtained in a previously published study the rat L-NAME model [12].

L-NAME model. The hypertensive state in the rats was induced by adding the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) in the drinking water at 400 mg/L. The rats received approximately 40 mg/kg/day of L-NAME.

Blood-pressure measurement. The blood-pressure was measured by using the tail-cuff method (CODA, Kent scientific corporations) following the manufacturer's instructions. Blood-pressure levels were measured at baseline and after 2 and 4 weeks of intervention/treatment. Both systolic (SBP) and diastolic blood pressure (DBP) were measured.

Blood-sample analyses. Serum and plasma samples were taken at the end of the experiment by heart puncture and were centrifuged within 30 min (2 200 g for 10 min at 4°C). All samples were stored at -70°C until analysis.

Body and organ weights. The rats were weighed at baseline and then after 2 and 4 weeks of treatment prior to blood pressure measurement. At the end of the study, after the rats had been anesthetized and heart punctured, the heart, spleen, liver and one kidney were removed and weighed.

Caecal microbiota analyses. Terminal restriction fragment length polymorphism (T-RFLP) and SYBR green quantitative polymerase chain reaction (qPCR) were used to analyse caecal microbiota diversity and composition.

T-RFLP analysis. Genomic DNA was extracted from the caecal content with EZ1 DNA tissue kit (Qiagen, Hilden, Germany) on a BioRobot EZ1 workstation (Qiagen) according to the manufacturer's instructions. The 16S rRNA gene was amplified by using a fluorescently labeled forward primer ENV1 (5'-FAM-AGAGTTTGATIITGGCTCAG-3') and a non-labeled reverse primer ENV2 (5'-CGGITACCTTGTTACGACTT-3'). The PCR reaction was performed in a total volume of 25 µL consisting of 0.4 µM of FAM-ENV1 primer and 0.2 µM of primer ENV2, 2.5 µL of 10x TopTaq PCR buffer (containing 15 mM $MgCl_2$), 0.2 mM of each dNTP, 1.25 U of TopTaq DNA polymerase (Qiagen), and 10–20 ng of template DNA. The PCR was performed under the following condition: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 50°C for 45 s, and 72°C for 2 min. An additional extension at 72°C for 7 min was done at the end. All PCR reactions were carried out in triplicate and pooled for each sample. The PCR products were purified with QIAquick PCR Purification Kit (Qiagen) and quantified using NanoDrop ND-1000 (Saveen Werner, Limhamn, Sweden). Then, 200 ng of the purified DNA was digested with the endonuclease *Msp*I (Fermentas Life Science, Burlington, Canada) in a total volume of 10 µL according to the manufacturer's instruction. The resulting fragments were analysed on an ABI 3130xl Genetic analyzer (Applied Biosystems, Foster city, CA, USA) with internal size standard GeneScan LIZ 600 (Applied Biosystems) at DNA-lab (SUS, Malmö, Sweden). The T-RFLP profile was analysed using the GeneMapper software version 4.0 (Applied Biosystems). The relative area percentage was calculated for each T-RF and used for diversity calculation and principal component analysis (PCA).

SYBR green qPCR. Bacterial stains used for constructing plasmid standard curves were *L. plantarum* DSM 9843, *Bifidobacterium infantis* DSM15159 (*B. infantis* CURE 21; Probi AB, Lund, Sweden), *Escherichia coli* CCUG 29300, *Bacteroides fragilis* ATCC 25285, *Clostridium leptum* DSM753, and *Desulfovibrio desulfuricans* subsp. *desulfuricans* CCUG 34226. The detailed procedure has been described elsewhere

[13]. The qPCR reaction was performed using Rotor-Gene SYBR Green PCR kit (Qiagen) in a real-time PCR cycler Rotor-Gene Q (Qiagen). The PCR reaction was prepared in 20 µl volume consisting 10 µl of 2× Rotor-Gene SYBR Green PCR Master mix, 0.5 µM of each primer and 2 µl of template DNA. Duplicate reactions were run for each sample, standard and negative controls. The primer sequences were listed in Table 1. The qPCR condition was initiated with activation at 95 °C for 5 min followed by 40 cycles at 95°C for 5 s, and combined annealing and extension at 60°C for 10 or 15 s. Melt curve analysis was performed for each run.

Statistical analysis. Student's t-test was used for the statistical analysis of the results comparing the differences between treated and control animals. Bar graphs represent means ± standard deviations (SD) of the corresponding values. Differences were considered significant if $p < 0.05$. For the data that were not normally distributed, including diversity indices and qPCR data, the Wilcoxon-Mann-Whitney rank sum test was used for comparisons between the treated and control group using the package "coin" in the R program (version 2.15.1) Values of $P < 0.05$ were considered statistically significant. T-RFLP data were analysed with principal component analysis (PCA) using SIMCA-P software version 12.0.1.0 (Umetrics, Umeå, Sweden).

Ethical approval. The study protocol was approved by the local ethical committee at the University of Lund.

Results

Effect of fermented blueberries and probiotic bacteria on blood pressure. To avoid the effect of baseline differences in the systolic and diastolic blood pressure among the six study groups, the results at each time point are presented as difference in mmHg from baseline values. Consumption of L-NAME in the drinking water for two weeks significantly increased the blood pressure (SBP/DBP) by approximately 32 %, from 139 ± 6.5/96 ± 8.3 mmHg to 182 ± 10.7/128 ± 11.2 mmHg (Fig. 1a and 1b). Intake of the fermented blueberry product B with probiotics (group LN+B) significantly reduced both the systolic and diastolic blood pressure elevation that was induced by L-NAME within the 2-week period ($p < 0.004$ for SBP and $p < 0.008$ for DBP). The induced SBP and DBP were also reduced by blueberry product A (group LN +A), however, not at levels of statistical significance ($p < 0.19$ for SBP and $p < 0.075$ for DBP). In contrast, product A significantly reduced the SBP ($P < 0.008$) and DBP ($P < 0.013$) of rats that had not been given L-NAME (non-L-NAME-treated rats) in their drinking water, after 4 weeks of intervention (group W + A). This effect on the non-L-NAME treated rats was not observed with product B (group W + B).

Differences in feed and water consumption among groups. It was observed that whereas product B was consumed to 100 % and 98 % by the rats in groups W+B and LN+B respectively, product A was consumed to 96 % by group W+A and to 85,6 % by LN+A (Fig. 2a). It was also observed that the rats eating product A were drinking more water when compared to the other groups, as described in Fig. 2b. No differences in water consumption were detected for the other four animal groups (cont W, W+B, cont LN, LN+B) that consumed approximately 30 ml water/rat daily.

Effects on body weight. No baseline differences were detected in body weight among the animals of the different study groups. All rats continued to gain weight and looked healthy during the first 3 weeks of the study. A few days before the end of the study it was observed that some of the animals among L-NAME treated rats, i.e. groups cont LN (1 rat), LN+A (3 rats) and LN+B (3 rats) started loosing weight and showed reduced physical activity in their cages.

Effects on organ weights. No major differences were detected for the heart, kidney and spleen among the animal groups in the study other than a trend towards increased spleen weight after treatment with L-NAME (Fig. 3a). However, the liver of non-L-NAME rats eating product A (group W+A) weighed significantly less than the non-L-NAME rats (cont W) eating standard chow (Fig. 3b). When the organ weights were recalculated per body weight to take into consideration the differences in total body weight

gain towards the end of the study the spleen weight was increased in some of the study groups (Fig. 3c). Intake of product A (groups W+A and LN+A) and product B (group LN+B) was associated with an increased ratio of mg spleen/g body weight. In a similar way the combined intake of L-NAME and product A or product B was associated with an increased ratio of heart weight/body weight (Fig. 3c). The liver in group W+A weighed less than the control (cont W) even when calculated in mg liver/g body weight ($p < 0.055$, Fig. 3d).

Blood sample analyses. Alanine aminotransferase (ALAT) and creatinine were analysed to check for possible effects of the treatment/diets on the function of liver and kidneys, respectively. It was observed that L-NAME seemed to affect the liver function and caused increased levels of ALAT in the L-NAME treated control (cont LN) as compared to the non-L-NAME treated animals ($p < 0.002$, Fig. 4a). Intake of product A significantly reduced the levels of ALAT in group W+A ($p < 0.037$), compared to the cont W group, as described in Fig. 4a. Fibrinogen, an inflammatory marker, was significantly increased in the L-NAME treated groups receiving product A ($p < 0.0005$) or product B ($p < 0.042$, Fig. 4a) compared to the cont LN group. No differences were detected in the levels of creatinine between the study groups (Fig. 4b). Analysis of various blood lipids revealed that cholesterol and low density lipoprotein (LDL) were increased in L-NAME treated rats receiving product A as compared to non-L-NAME treated rats cont W ($p < 0.009$ and $p < 0.015$ respectively, Fig. 4c). An increase was also observed for high density lipoprotein (HDL) in L-NAME treated rats given product A, however not at statistically significant levels ($p < 0.087$, Fig. 4c). L-NAME treated rats given product B showed significantly increased levels of triglycerides compared to both cont W and cont LN ($p < 0.004$ and $p < 0.029$ respectively, Fig. 4c).

Effects on caecal microbiota. The ceacal microbiota of non-L-NAME treated rats was mapped in order to search for a link between reduced BP and change in the composition of the microbiota. T-RFLP analysis showed that the diversity estimated by T-RF richness, Shannon's and Simpson's diversity index, didn't differ between the groups (Table 2). PCA analysis of the T-RFLP profile revealed that caecal microbiota was affected by consumption of product A and B (Fig. 5). However, there was no clear separation between the groups W+A and W+B. The major driving force for the separation between cont W and product receiving groups is the change in T-RF 303, 228, 132, 91 and 92. The abundance of T-RF 303 and T-RF 228 was generally decreased in W+A and W+B (means 10.7% vs. 3.7% vs. 1.7% of T-RF 303; 14.5% vs. 6.7% vs. 7.1% of T-RF 228). T-RF 132 was only detected in W+A and W+B group and interestingly three animals housed in one cage from W+A group had high abundance (around 20%) of this bacterial group represented by T-RF 132. T-RF 91 and 92 were found in all animals and had moderate increase in groups W+A and W+B (means 7.4% vs. 11.2% vs. 13.8% of T-RF 91; 8.3% vs. 10.7% vs. 9.5 % of T-RF 92).

Quantification of certain bacterial groups by qPCR (Table 3) showed that *Clostridium leptum* group was significantly decreased in W+B group ($p=0.02$) but not in W+A group ($p=0.07$) compared to cont W. The amount of *Desulfovibrio* was significantly decreased in W+B compared to cont W. The load of *Enterobacteriaceae* was lower in the product-receiving groups but did not reach statistical significance (W+A vs. cont W, $p=0.1$; W+B vs. cont W, $p=0.08$). No significant change was found in either amount of *Lactobaicllus* or of the *Bacteroides fragilis* group. Bifidobacteria were detected in 7 out of 9 rats in cont W (median 7.46 log copy/g), in 3 out of 9 rats in W+A (median 7.13 log copy/g) and in 4 out of 9 rats in W+B (median 7.97 log copy/g).

Discussion

The principal aim of the present study was to examine the anti-hypertensive capacity of a probiotic product consisting of blueberries fermented by *L. plantarum* DSM 15313. The chosen animal model of L-NAME treated rats is commonly used [14] and has previously been applied to study the effects of various substances such as piperine, red wine polyphenols or *Yucca schidigera* extract on blood pressure [15-18]. In the present study we showed that freeze-dried fermented blueberries significantly reduced the L-NAME induced hypertension within two weeks of treatment. The results were significant both for the systolic and

the diastolic blood pressure ($p < 0.004$ and $p < 0.008$ respectively, Fig. 1). Furthermore, within four weeks of treatment there was a significantly lower blood pressure in non-L-NAME treated rats receiving a fermented blueberry product as compared to the control group ($p < 0.008$ for SBP and $p < 0.013$ for DBP, Fig. 1). However, this effect was mainly obtained with product A and to a lesser extent with product B which, most probably, emphasizes the importance of the higher phenolic acid content in product A. Indeed the anti-hypertensive capacity of a mixture of the three phenolic acids found at higher concentrations in product A compared to product B (hydroxyphenyllactic acid, 3,4-dixydroxyphenyl-propionic acid and phenyllactic acid) , had previously been examined and confirmed in L-NAME treated rats [12]. However, comparing the effect on blood pressure obtained with the phenolic acid mixture to that obtained with the intact whole fermented blueberries, it appeared as if other components too in the latter product, other that the above mentioned phenolic acids, contributed to the achieved effect (non-published data, Irini Lazou Ahrén). It could be that quercetin, a flavonol found in blueberries, accounted for the additional anti-hypertensive effect obtained with the whole blueberry product [19, 20].

A change in caecal microbiota was observed in the rats given product A or B in the non-L-NAME treated groups. Intake of product A and B led to an obvious decrease in the abundance of T-RF 303, which was putatively identified as bacteria belonging to *Lachnospiraceae* in another study that used the same rat strain (non-published data, Jie Xu). It could be speculated that the *Lachnospiraceae* were susceptible to the antimicrobial activity of the blueberries. On the other hand, the abundance of T-RF 88, 91, 92 which were identified as *Parabacteroides*-like and *Bacteroides*-like (non-published data, Jie Xu), were increased in W+A and W+B. Similar observations were reported in a rat model where *Bacteroides* species were found to be increasing with tannin-diet, whereas the level of *Clostridium leptum* group was decreasing [21]. In the current study, qPCR result showed a decreasing trend in the load of *Clostridium leptum* group with the blueberry product supplementation while *Bacteroides fragilis* group were not significantly affected. Thus, other *Bacteroides* species seem to have contributed to the increase in the *Bacteroides*-like T-RFs. It has been shown that rat fecal microbiota can release quercetin [22], and some of the *Bacteroides* species can metabolize the quercitrin (glycosylated form of quercetin) to quercetin [23]. Further studies are needed to confirm whether the increase in the population of *Bacteroides* related species in W+A induced an increase in quercetin bioavailability. The PCA analysis of the T-RFLP data revealed that the supplemented product A and B had an impact on caecal microbiota. However, no clear difference in bacterial composition could be seen between the two groups. This observation indicates that the higher level of phenolic acids in product A did not exert a strong selection pressure on the caecal microbiota. In addition, large variations in the structure of the caecal microbiota between the individual rats were seen in each group. Animals housed in the same cage shared more similar microbiota than the ones from different cages, suggesting there was a cage-effect that contributed to the large variation. To better see the effects of the phenolic compounds or other active substances from the tested products on the intestinal bacteria, effort in increasing the homogeneity of the microbiota composition may be necessary in the future.

Product B, was more efficient in inhibiting the rise in the systolic and diastolic blood pressure of L-NAME treated rats when compared to product A. The systolic blood pressure was reduced by 46 % with product B ($p < 0.004$) and by 20.9 % with product A ($p < 0.1873$) within two weeks. A possible explanation for the difference in effect between the two study products could be the fact that the rats in group LN+A consumed more L-NAME-containing water but less feed and consequently less study product than the rats in group LN+B (Fig. 2). This could in theory result in a less efficient and not statistically significant inhibition of the blood pressure elevation. The successful reduction by products A and B of the induced blood pressure in the L-NAME treated groups reveal that the fermented blueberries in combination with *L. plantarum* DSM 15313 are capable of affecting the blood pressure at least through one mechanism that involves a nitric oxide (NO)-dependent pathway. Moreover, the blood-pressure lowering effect by products A and B in the animals of the non L-NAME treated rats could imply the existence of other NO-independent mechanisms supporting the anti-hypertensive activity of the fermented blueberry products.

To our knowledge, this is the first time the anti-hypertensive activity of blueberries is shown in healthy rats with normal blood pressure. The anti-hypertensive activity of berries has previously been studied in clinical trials and animal models [24]. Intake of a probiotic product for 6 weeks decreased the blood pressure in healthy smokers from 134 ± 20 mmHg to 121 ± 16 mmHg as compared to the placebo where

the blood pressure changed from 128 ± 18 mmHg to 126 ± 16 mmHg [25]. Furthermore, in the same study, intake of probiotics reduced inflammatory markers such as fibrinogen and leptin, known to be associated with an increased risk for cardiovascular diseases. It was hypothesized in that study that the combination of tannin-rich rosehip and a probiotic with tannase activity resulted in the production of phenolic acid compounds with anti-inflammatory properties. Focusing on blueberries, it has been reported that daily intake of 50 g freeze-dried blueberries for 8 weeks reduced both the systolic and diastolic blood pressure in obese men and women with metabolic syndrome [26]. Spontaneously hypertensive stroke-prone rats eating a diet enriched with 3% freeze-dried blueberries for 8 weeks had their systolic blood pressure reduced by 30% [27]. Other reported effects of blueberries on risk factors for cardiovascular diseases and the metabolic syndrome include reduced insulin sensitivity, increased anti-oxidant activity and reduced lipid peroxidation [28-30]. Furthermore, it was recently published that an 8% wild blueberry-enriched diet improved the vascular tone in adult spontaneously hypertensive rats with endothelial dysfunction [31]. The same group had earlier reported that in Sprague Dawley rats diets enriched with wild blueberries seemed to affect NO metabolic pathways in the aorta at basal and stimulated levels [32].

L. plantarum DSM 15313 and freeze-dried blueberries were shown to reduce the levels of the enzyme alanine aminotransferase (ALAT) in a D-galactosamine and LPS-induced liver injury model [33]. This is in accordance with the results obtained in the present study. Product A significantly reduced the levels of ALAT in the animal group W+A after four weeks of treatment ($p < 0.002$, Fig. 4a).

It was observed that towards the end of the study some of the L-NAME treated animals lost weight. However, since none of the animals in the three non-L-NAME treated groups showed any weight loss we attributed this effect to the animal model itself and not the study products. Furthermore, it is difficult to explain the increased lipid levels in the L-NAME treated groups (Fig. 4c). However, we find that the most important results in the present study are the ones obtained with normal control rats that are not under the effect of any induced "abnormal" situation with broader consequences for their health.

To summarize, in the present study we report that live *L. plantarum* DSM 15313 and blueberries that have been fermented by the same bacterial strain have the ability to lower both the systolic and diastolic blood pressure of both non-L-NAME and L-NAME treated rats. In addition, the tested products altered the gut microbiota of the non-L-NAME treated rats, i.e. in non-manipulated healthy rats. In theory, such a product could potentially reduce the risk for cardiovascular diseases and the metabolic syndrome. The next step would be to confirm this effect in a clinical trial recruiting participants with a high blood pressure. Functional food with anti-hypertensive activity would help millions of people worldwide to either reduce their high blood pressure or prevent a normal-high blood pressure from reaching levels of hypertension that require medication.

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Conflict of interest The authors Irini Lazou Ahrén and Gunilla Önning are employed at Probi AB.

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Table 1. Primers used in SYBR green qPCR for bacterial quantification.

Name	Sequence (5'—3')	Target group	Reference
Lact-16S-F	GGAATCTTCCACAATGGACG	*Lactobacillus*	[34]
Lact-16S-R	CGCTTTACGCCCAATAAATCCGG		
Bif-F	TCGCGTCYGGTGTGAAAG	*Bifidobacterium spp*	[35]
Bif-R	CCACATCCAGCRTCCAC		
Eco1457-F	CATTGACGTTACCCGCAGAAGAAGC	*Enterobacteriaceae*	[36]
Eco1652-R	CTCTACGAGACTCAAGCTTGC		
g-Bfra-F	ATAGCCTTTCGAAAGRAAGAT	*Bacteroides fragilis group*	[37]
g-Bfra-R	CCAGTATCAACTGCAATTTTA		
Clept-F	GCACAAGCAGTGGAGT	*Clostridium leptum* group	[37]
Clept-R	CTTCCTCCGTTTTGTCAA		
DSV691-F	CCGTAGATATCTGGAGGAACATCAG	*Desulfovibrio*	[38]
DSV826-R	ACATCTAGCATCCATCGTTTACAGC		

Table 2. Diversity indices calculated based on T-RFLP data.

	cont W		**W+A**		**W+B**	
	Median	*25-75 %*	**Median**	*25-75 %*	**Median**	*25-75 %*
Richness	**29**	*23-31*	**31**	*26-33*	**31**	*30-32*
Shannon's index	**2.82**	*2.69-2.90*	**2.89**	*2.69-3.01*	**2.98**	*2.87-3.02*
Simpson's index	**0.92**	*0.89-0.92*	**0.92**	*0.88-0.93*	**0.92**	*0.92-0.93*

Table 3. Quantification of cecal bacteria by SYBR green qPCR

	cont W		**W+A**		**W+B**	
	Median	*25-75 %*	**Median**	*25-75 %*	**Median**	*25-75 %*
Lactobacillus (log copy/g)	**8.12**	*7.98-8.69*	**8.14**	*7.63-8.32*	**8.32**	*8.23-8.46*
Enterobacteriaceae (log copy/g)	**9.97**	*9.32-10.33*	**9.16**	*8.34-9.78*	**9.23**	*8.91-9.32*
Bacteroides fragilis group (log copy/g)	**9.68**	*9.44-9.88*	**9.92**	*9.74-10.24*	**9.80**	*9.55-10.10*
Clostridium leptum group (log copy/g)	**9.14**	*8.78-9.24*	**8.51**	*7.47-9.05*	**8.61***	*7.0-8.64*
Desulfovibrio (log copy/g)	**7.06**	*6.98-7.15*	**6.99**	*6.70-7.26*	**6.73***	*6.43-6.97*

*denotes $p<0.05$ compared to cont W.

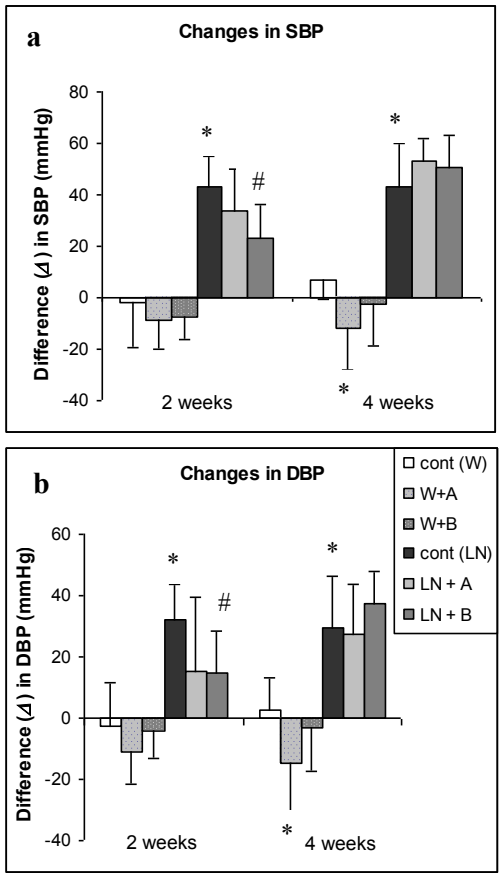

Fig. 1 Mean ± SD changes in systolic (SBP; a) and diastolic (DBP; b) blood pressure in the control and L-NAME treated rats with or without supplementation of fermented blueberry products A and B. Results are shown for 2 and 4 weeks of product administration. Significance is set at $p<0.05$. * means significantly different from the non-L-NAME treated control rats (cont W) and # means significantly different from the L-NAME treated control rats (cont LN).

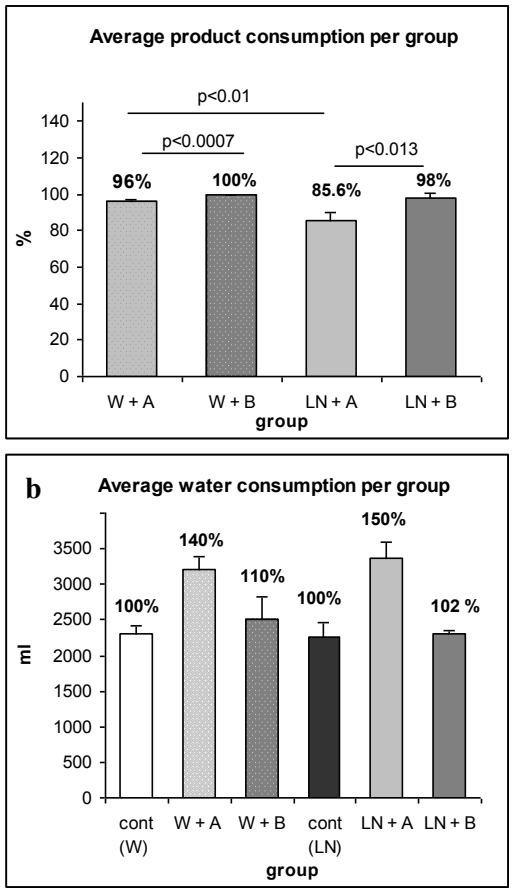

Fig. 2 Average consumption of food (a) and water (b) per group receiving study products A or B. Results are shown in percentage for the food and in ml for the water and represent consumption as compared to the total amount supplemented per group. Significance is set at $p<0.05$

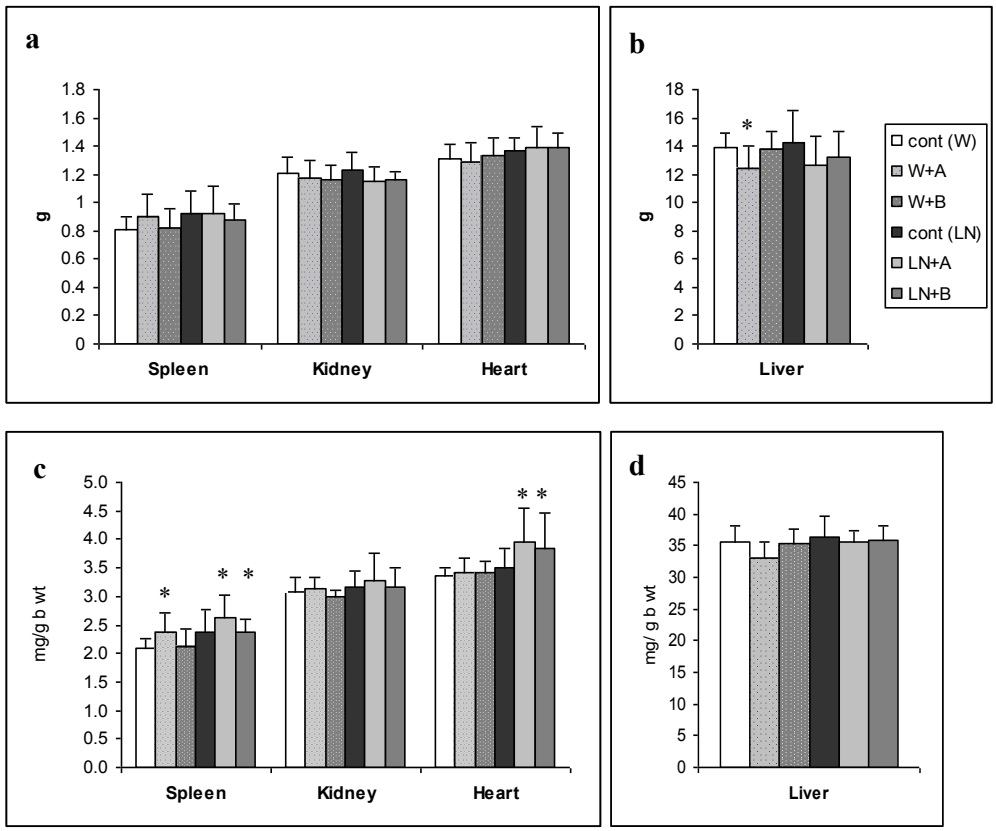

Fig. 3 Mean ± SD changes in organ weights after four weeks of intervention. The results are shown either in g organ weight (fig. 3a for spleen, kidney, heart and 3b for liver) or in mg organ weight per g total body weight (fig. 3c for spleen, kidney, heart and 3d for liver). Significance is set at $p<0.05$ and * means significantly different from the non-L-NAME treated control rats (cont W).

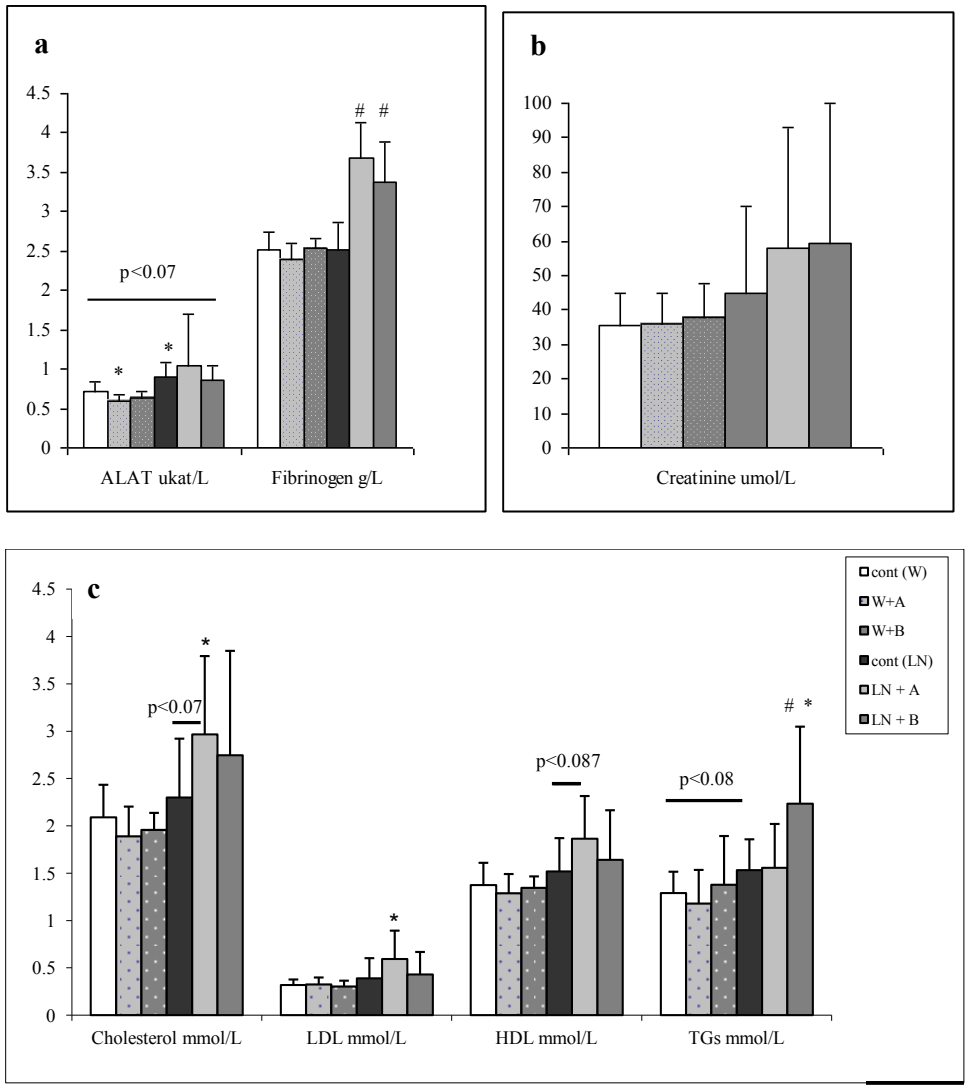

Fig. 4 Mean ± SD values for various blood markers after 4 weeks of intervention. a): results shown for the liver enzyme alanine aminotransferase (ALAT) in kat/L and for the inflammatory marker fibrinogen in g/L. b): results shown for creatinine (mol/L), a marker for kidney function. c): blood lipid analysis showing results in mmol/L for cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides (TGs). Significance is set at $p<0.05$ and * means significantly different from the non-L-NAME treated control rats (cont W) and # means significantly different from the L-NAME treated control rats (cont LN).

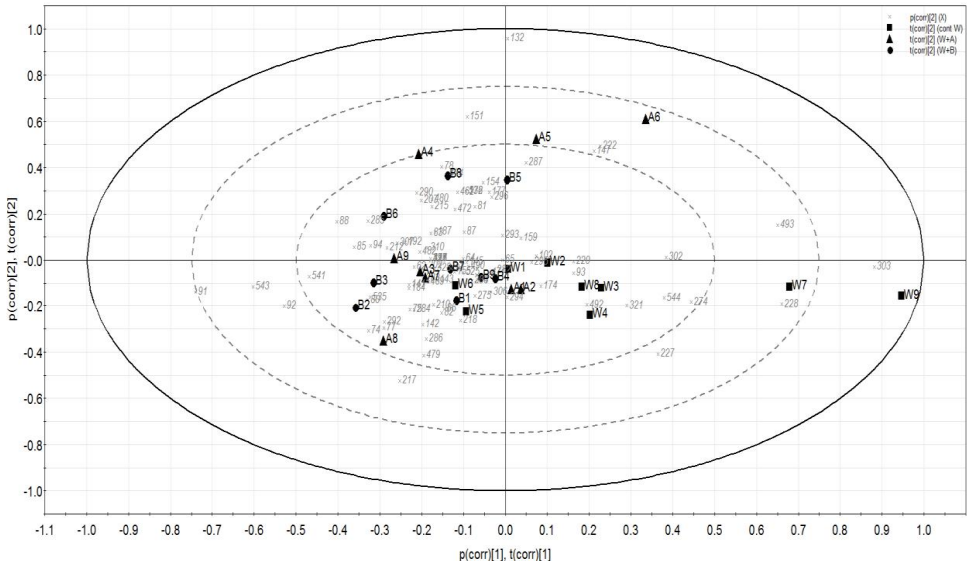

Fig. 5 PCA loadings biplot. Boxes (cont W): non-L-NAME treated control rats; triangles (W+A): non-L-NAME treated rats receiving product A and bacteria; dots (W+B): non-L-NAME treated rats receiving product B and bacteria. W1-9, A1-9 and B1-9 indicate 9 rats included in cont W, W+A and W+B group respectively. Rat numbered 1-3, 4-6, and 7-9 were housed in one cage in each group. The numeric numbers indicate detected T-RFs (bases) representing different bacterial groups.

Paper III

Oral and fecal microbiota in volunteers with hypertension in a placebo controlled trial with probiotics and fermented bilberries

Jie Xu*[1], Irini Lazou Ahrén[2], Crister Olsson[3], Bengt Jeppson[4], Siv Ahrné[1], Göran Molin[1]

[1]Laboratory of Food Hygiene, Department of Food Technology, Engineering and Nutrition, Lund University, Box 124, SE-221 00 Lund, Sweden

[2]Probi AB, Lund, Sweden

[3]Department of Biosystem and Technology, Swedish University of Agricultural Science, P.O. Box 103, 230 53 Alnarp, Sweden

[4]Surgery Research Unit, Department of Clinical Science, Lund University, Skåne University Hospital, 205 02 Malmö, Sweden

*Corresponding author. Email: Jie.Xu@appliednutrition.lth.se, Phone: +46-46 222 98 16

Abstract

Background. Research on the microbiota of the digestive tract in recent years suggest a link between microbiota and metabolic syndrome such as obesity and type 2 diabetes, thereby pointing at the microbiota as a new target for the prevention and treatment of these lifestyle related diseases. Hypertension is one common symptom in people with metabolic syndrome. However, few studies have looked at the relationship between the intestinal microbiota and hypertension. Probiotics, prebiotics or synbiotics are means to modulate the microbiota. In this study, we tested two food supplements containing either live *Lactobacillus plantarum* HEAL19 (HEAL19) or a mixture of HEAL19 together with fermented bilberries by the same bacterial strain for anti-hypertensive potentials.

Results. Our randomized, double-blind, placebo-controlled study showed that consumption during 3 months of the tested supplements did not reduce the blood pressure in adult people with hypertension. Analysis on the oral and fecal microbiota using terminal restriction fragment length polymorphism (T-RFLP) and 454 amplicon pyrosequencing showed that the microbiota diversity and composition was not affected by the supplements and remained relatively stable throughout the study period. Rare species found by higher sequencing depth had little impact on the diversity. There was no clear correlation between the oral and the fecal microbiota. The oral one fluctuated more with time and showed a much larger individual variation than the fecal microbiota. *Bacteroides* and unclassified *Rikenellaceae* were found to be the two most fluctuating major taxa in the fecal microbiota that was otherwise relatively stable. *Akkermansia* was detected in feces of 43 out of 90 individuals and showed large variation between individuals regarding the change in abundance by time. No distinct enterotypes could be identified.

Conclusions. *L. plantarum* HEAL19 or a mixture of HEAL19 together with fermented bilberries did not affect oral or fecal microbiota in volunteers with hypertension. The overall structure of the oral and fecal microbiota was stable over 3 months. *Bacteroides* and unclassified *Rikenellaceae* were found to be the two most fluctuating major taxa in the fecal microbiota that was otherwise relatively stable.

Trial registration at ClinicalTrials.gov with following number: NCT01989702

Key words: microbiota; blood pressure; *Lactobacillus plantarum* HEAL19; bilberry

Introduction

The microbiota of the gastrointestinal (GI) tract has been shown to be associated with the conditions of metabolic syndrome such as obesity and type 2 diabetes [1-4]. However, the link between microbiota and hypertension or high blood pressure, which is another common component of the metabolic syndrome, has not yet been fully identified. Hypertension defined as systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg is a major risk factor for cardiovascular disease. Modifications in sedentary lifestyle and diet pattern are recommended for prevention and treatment of hypertension [5]. In recent years, probiotics gained growing interests from researchers for its potentials to convey health benefits. Consumption of dairy products containing probiotic bacteria showed antihypertensive effect via production of inhibitory peptides against angiotensin-I-converting enzyme during fermentation process [6, 7]. However, the effect of these living bacteria on the resident bacteria was not analyzed.

The human GI tract harbors a complex and diverse microbial ecosystem that is involved in the digestion and nutrient absorption, essential vitamin synthesis and development of immune system [8-11]. The acquisition of the gut microbiota is thought to start during birth when the infant comes in contact with maternal vaginal and fecal microbiota. However, there is also evidence showing that colonization of the gut microbiota could begin before birth. The inter-individual variation is large between babies and multiple factors including maternal gut microbiota, delivery mode, breast feeding, genetic and environmental exposures are usually involved in the shaping of the early gut microbiota [12-14]. After the first colonization the gut microbiota will continue to mature and become more diverse till around the age of 3 when it starts to resemble the adult microbiota and the composition becomes relatively stable [12, 15]. The variation in composition usually is smaller within the person over time than between different individuals [16-18]. The stability of adult gut microbiota both in the diversity and composition is maintained until old age (~70 years). However, a shift in diversity and microbiota composition seem to occur later in life, i.e, centenarians harbored a microbiota with a decreased diversity and enrichment of bacteria with pathogenic potential [19]. Even in a shorter time span, when tested after 24 hours and over 3 months, the stability of healthy individuals' GI microbiota was maintained [17] .

Since the human GI microbiota seems to be relatively stable over time within a person and that a high variation exists between individuals it may bring a challenge for probiotic interventions aiming to achieve health beneficial effects through the regulation of the microbiota composition. Along the GI tract, microbiota associated with different habitats varies dramatically [17], which in turn may lead to different responses to probiotic stimuli. Intake of probiotics can affect the oral microbiota as well as the microbiota of the small and large intestine.

Diets rich in fruits and vegetables are recommended for the prevention and treatment of hypertension [20]. It has been reported in both animal models and human studies that consumption of antioxidant- rich blueberries could lower blood pressure [21-25]. Among various species of blueberries (*Vaccinium* spp.), the European blueberry or bilberry (*Vaccinium myrtillus*) has the highest content of anthocyanins [26].

The current study, primarily aimed to test the anti-hypertensive effect of a non-milk based food supplement which contained live *Lactobacillus plantarum* HEAL19 alone or together with fermented bilberries. The composition and stability of both the oral and the fecal microbiota in adult participants were followed during a study period of three months.

Materials and Methods

Participants and study design. In total, 142 participants were included in this double-blind placebo controlled study, after written informed consent, according to the following inclusion criteria: i) healthy individuals at the age of 40-75 years, ii) blood-pressure >140/90 mmHg, iii) triglycerides >1.7 mmol/L and/or HDL <1 mmol/L (men)/1.29 mmol/L (women), iv) fasting plasma glucose levels >5.6 mmol/L and/or

waist circumference >102 cm (men)/88 cm (women). Ongoing medication for high levels of blood lipids excluded people from participating in the study.

The study consisted of a 2-week "run-in period (day 1-14) and a 12-week intervention period during which the subjects were taking the probiotic study products or placebo once daily (day 15-98). All study participants were asked to refrain from consumption of other products containing probiotic bacteria during their participation in the study. Exclusion criteria at the time of enrolment were: medically treated allergy or allergy to any of the ingredients of the study product, medication for high levels of blood lipids, presence of metabolic disease, such as type one diabetes, confirmed disease of the heart, liver or kidneys, chronic inflammatory disease requiring medication, pregnant or nursing, regular intake of a probiotic product (5-7 days per week) during the last three months before inclusion into the study.

The study was conducted in accordance with the Declaration of Helsinki and ethical approval was given by the Ethics Committee at the University of Lund, Sweden (Dnr 2009/66).

Intervention. Participants were randomly allocated to receive either one of the two probiotic products or placebo. The study product was a combination of a fruit drink with or without fermented bilberries and a powder consisting of maltodextrin with or without the probiotic bacteria *Lactobacillus plantarum* HEAL 19 (DSM 15313). The participants in the probiotic group (P) received placebo drink and placebo powder. The participants in the fermented bilberry group (BL) received fruit drink with fermented bilberries, corresponding to approximately 10 g fresh bilberries/day and placebo powder. The third group received placebo drink and active probiotic powder containing 1 x 10^9 cfu *Lactobacillus plantarum* HEAL 19 (DSM 15313)/daily dose. Participants were instructed to daily drink 150 ml of the fruit drink mixed with the powder from one sachet. They were further instructed to drink the study product at the same time every day, if possible. Mouth swabs and fecal samples were taken at the beginning and the end (after 3months) of the study.

Blood pressure measurements. All measurements were according to the guidelines by the European Society for Hypertension for the management of arterial hypertension [27] and were done using an automatic blood pressure measuring machine. The blood pressure was measured 1-2 times during the run-in phase, just before the start of intervention and at 4-8-12 weeks (study weeks 6-10-14). The blood pressure was measured in a sitting position after at least 5 minutes of rest. The cuffs were sitting on the arm at the level of the heart. The mean of at least two measurements with no less than 2 minutes in between were taken.

DNA extraction. The fecal samples were lyophilized prior to the DNA extraction and the mouth swab samples were centrifuged at 10,000×g for 5 min and the supernatant was discarded. Twenty mg of the lyophilized fecal samples and the pellets from the mouth swabs were used to extract the genomic DNA with EZ1 DNA tissue kit (Qiagen AB, Sollentuna, Sweden) on a BioRobot EZ1 workstation (Qiagen), respectively as described elsewhere [28].

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis. The 16S rRNA gene was amplified by forward primer ENV1 (5′-AGAGTTTGATIITGGCTCAG-3′) and reverse primer ENV2 (5′-CGGITACCTTGTTACGACTT-3′). The 5′ end of the forward primer was fluorescently labeled with 6'-carboxyfluorescein (FAM) dye. The PCR reactions were carried out with triplicates followed by pooling and purification. The purified 16S amplicons were digested with endonuclease *Msp*I and analyzed on an ABI 3130xl Genetic analyzer (Applied Biosystems, Foster city, 212 CA, USA) at DNA-lab (SUS, Malmö, Sweden). The resulting T-RFLP data was analyzed with the Genemapper® software version 4.0 (Applied Biosystems) with local southern algorithm. Details of PCR conditions, purification steps and digestion settings are described as elsewhere [28].

Pyrosequecing of 16S rDNA amplicons (V1-V3). Twelve fecal samples taken from six volunteers at the beginning of the study and after 3 months were selected for pyrosequencing. Three people had elevated BP and the other three had decreased BP after three months. These six people had big changes in the T-RF 91-93

(Table 1). The V1-V3 regions of the 16S rRNA gene was amplified with forward primer 5′-MIDx-AGAGTTTGATIITGGCTCAG-3′ and reverse primer 5′-MIDy-GWATTACCGCGGCKGCTG-3') where MID is a unique multiplexing identifier composed of 10 nucleotides that are used for barcoding the sequences. The MIDs are different for the forward and reverse primers. The PCR reactions were prepared in triplicates and the amplicons were purified with MinElute Gel Extraction kit (Qiagen). The PCR cycling conditions were 94°C for 3min, followed by 30 cycles of 94°C for 30 s, 54°C for 40 s and 72°C for 1 min. An additional extension at 72°C for 10 min was performed at the end of PCR. The concentration of DNA was measured by Nanodrop ND-1000 (Saveen Werner, Limhamn, Sweden). Amplicons were pooled in equal amount to have a final concentration of 25 ng/µl. The PCR products were subsequently amplified by emulsion PCR and sequenced at MWG, Germany using GS FLX+ chemistry.

Sequence analysis. Sequences were analyzed with an open source software package Quantitative Insights Into Microbial Ecology (QIIME 1.7.0) [29]. First, two mapping files were created for forward and reverse reads. Then, with the split library script the sequences were filtered for sequence length > 200 nt, quality score window >50, having correct primer sequence, maximum length of homopolymer of 6 nt. Reverse primer sequence and any subsequence sequence was also removed. The filtered sequences were assigned to samples according to the barcodes. The reverse reads were reverse complemented and then concatenated with forward reads. Operational Taxonomic Units (OTUs) were picked with 97% sequence similarity using uclust [30]. Most abundant sequences found in each OTU was picked as representative sequences and aligned against Greengenes core set [31] using PyNAST [32] with a minimum sequence identity of 75% and a minimum sequence length of 300 nt. Taxonomy was assigned to the representative sequences using Ribosomal Database Project (RDP) Classifier with minimum confidence score of 0.8 [33]. Potential chimeras were identified with ChimeraSlayer [34] and removed from the aligned sequences. An OUT table was built with non-chimeric and non-PyNAST failure sequences and used for downstream analysis including alpha and beta diversity calculations, hierarchical clustering with Unweighted Pair Group Method with Arithmetic mean (UPGMA) and three-dimensional principal coordinate analysis (PCoA). Shannon's index was computed with log base 2 and thus recalculated to natural logarithm to compare with the values obtained from T-RFLP analysis.

Statistical analysis. The data are presented as means±SEM and means±SD, and analyzed with Excel 2007 (Microsoft Corp., Redmond, USA). A free software PAST (version 2.17b, http://folk.uio.no/ohammer/past/) was used to calculate Shannon's diversity index using T-RFLP data. The T-RFLP data was also analyzed with principal component analysis (PCA) with SIMCA-P software (version 12.0.1.0; Umetrics, Umeå, Sweden). Values of $P < 0.05$ were considered statistically significant.

Results

No antihypertensive effect of the product. In total, 142 subjects were included in the study. Three of them were excluded due to changes in their anti-hypertensive medication and three dropped off due to abdominal discomfort (two persons) or because of lack of time for participation (one person). Eventually, 136 participants finished the study and one person had sudden drop in the BP and thus was excluded from the data analysis. The demographic data and the blood pressure values at baseline for all the participants are presented in Table 2. Intake of *L.plantarum* HEAL19 alone or with the fermented bilberries for 3 months did not significantly change the blood pressure. Although SBP and DBP decreased slightly after 2 months, this change was not statistically significant (Fig 1, p=0.27 for P, p=0.47 for BL, p=0.14 for L).

T-RFLP analysis showed no change in diversity either by products or by time. Both mouth and fecal samples from ninety volunteers were included in the present study. In total 138 and 182 T-RFs were found in oral and fecal microbiota, respectively. Diversity indices did not show any significant change either after consumption of the studied products or by time (Table 3 and 4).

PCA analysis showed certain dominant T-RFs driven clustering of the oral and fecal microbiota. The changes in T-RF occurrence and abundance over 3 months were analyzed with PCA, which revealed that the consumption of any of the studied products did not have major impact on either oral or fecal microbiota

composition (Fig 2 A and D). However, regardless of the intake of the supplements, both the oral and the fecal microbiota could be clustered into five groups according to the bacterial composition (Fig 2 B, C, E and F). Individuals that clustered into the same group with oral microbiota did not necessarily share the similar fecal microbiota. For the oral microbiota, the T-RFs that separate individuals into different clusters are mainly from the changes in T-RF 491, 549 and 550. *Streptococcus* species could be one candidate for T-RF 549 and T-RF 550 (non-published data). The bacterial groups represented by T-RF 491 and 549 were highly abundant in the oral microbiota (before vs. after values 29.1±2.0% vs. 31.4±2.4% and 21.4±1.5% vs.22.4±1.7%). T-RF 550 was present in a lesser amount with before vs. after values 8.5±1.2% vs. 6.5±1.1%. None of the changes in these dominant T-RFs can be linked to the administration of the study products. As seen in Fig 2C, the group1 had increase in abundance of T-RF491 after 3 months while decrease in abundance of T-RF 549. In contrast, group 2 had a decrease in abundance of T-RF 491 but an increase in T-RF 549. Group 3 had major increase in abundance T-RF549 and moderate increase in T-RF 491. Group 4 and 5 had no change to slightly decrease in T-RF 491, while group 4 also had larger decrease in T-RF 549 compared to group 5. For the fecal microbiota, changes in the T-RF 91, 92 and 93 had mainly driven the clustering (Fig 2F). The bacterial groups represented by these three T-RFs were dominant through the 3 months time with before vs. after values 7.8±1.5% vs.7.8±1.6%, 10.0±1.7% vs. 11.5±1.6% and 10.4±1.8% vs. 7.3±1.4% respectively. Fig 2F showed that group1 had increased level of T-RF 92 but decreased in T-RF 91, which is opposite of group 3. On the other hand, Group 2 had decrease in the abundance of T-RF 93 while group 4 had an increase. Group 5 had moderate changes in the three dynamic T-RFs compared to other groups. Furthermore, 54 out of 90 individuals belonged to group 5 indicating 60% of the tested people had a stable fecal microbiota throughout the time. However, the oral microbiota of these 54 individuals did not show a similar stability. These individuals were found in all the five groups in the oral microbiota clustering (Fig 2C).

Differences between oral and fecal microbiota. Comparison between the oral microbiota and fecal microbiota was done on the combined T-RFLP dataset of oral and fecal samples. In total 264 T-RFs were detected, in which 21.2% of the T-RFs were found in both oral and fecal samples. Compared to the fecal microbiota, the oral microbiota showed more fluctuation over time and a much larger variation between individuals (Fig 3).

Pyrosequencing of 16S rRNA gene amplicons (V1-V3 region). We used T-RFLP to get an overview of the oral and fecal microbiota of all the participants and it showed that there were certain dominant bacterial groups that changed by time. Due to these changes the participants clustered into different groups. Considering that oral microbiota showed more compositional fluctuation, and that the fecal microbiota was comparably more stable, we chose to focus on identifying those dynamic bacterial species in the fecal microbiota. To get more detailed information about the microbiota composition, a subset of the participants were selected. Twelve fecal samples from six individuals of which three had decreased and three had elevated blood pressure after three months, and showed big changes in T-RF 91, 92 and 93 were chosen for pyrosequencing analysis (Table 1). To be able to compare the results of T-RFLP, which covered the variable regions V1-V3, we targeted the same regions for pyrosequencing.

In total, 159759 sequences were obtained from forward and reverse reads. Differences in the number of sequences were seen between the forward and reverse reads. To minimize the bias, we combined the forward and reverse reads for each sample and analyzed the sequences. The sequences were clustered into Operational Taxonomic Units (OTUs) with 97% similarity, and the chimeric sequences (2.2% of the total sequences) were removed. In the filtered sequences, three bacterial phyla were found to dominate (>1%); *Bacteroidetes* (54.2%), *Firmicutes* (29.5%) and *Verrucomicrobia* (14.6%). *Bacteroidetes* and *Firmicutes* were dominant in all the tested subjects over three months. However, *Verrucomicrobia* were absent or present less than 0.1% in two out of 6 subjects. There was no correlation between blood pressure change and the change in the abundance of fecal bacterial phyla (Fig 4).

The bacterial richness and Shannon's diversity index based on the observed unique OTUs showed large variation between tested subjects and sometimes within the same subject at different sampling timepoints (Figure 5). Two samples (2A and 6B) had less than 500 sequences and thus were not shown in Figure 5. Sampling of 5000 sequences yield a 3-5 times increase in the number of observed OTUs compared to the sampling of 500 sequences (Fig 5A). However, sampling of around 1000 sequences was sufficient to measure the bacterial diversity (Fig 5B) which indicates that rare species found by higher sequencing depth had little impact on the diversity. The Shannon's diversity indices calculated at a normalized sequencing depth of 1078 sequences were significantly higher in comparison with the ones calculated from T-RFLP (3.45±0.25 vs. 1.87±0.10, $p<0.001$).

When the fecal microbiota was compared between subjects, one subject (number 6) was excluded due to the low number of sequences (186 sequences) at one sampling occasion. The microbiota structures were most similar within the same person over three months; however, subject number 5 showed large change in the microbiota between the start and 3 months later (Fig 4 and 6). This person had a sharp drop in diversity after three months (4.48 to 2.70) and the most obvious change was a dramatic increase of *Bacteroides* from 11% to 69% of the fecal microbiota after 3 months. On the other hand, for this person, the Shannon's diversity index calculated from the T-RFLP data did not show any obvious change but the abundance of the T-RF 91 increased 34% after 3 months. Interestingly, this person had dramatic increase in SBP and DBP by 49 mmHg and 20 mmHg, respectively. The ten major bacterial groups responsible for the separation between the subjects and within the subjects over three months are illustrated in Figure 7. One thing to note here is that the family named *"Barnesiellaceae"* belonging to order *Bacteroidales* is not an approved bacterial name, but a suggested change in taxonomy in the greengenes database (http://greengenes.lbl.gov) based on genome trees. These sequences were found abundant in subject number 4 (44.8% at the beginning and 16.2% after three months). For simplicity, genus *Barnesiella* was used to match the corresponding T-RFs.

Matching of T-RFLP and Pyrosequencing results. The pyrosequencing of the fecal samples, which were chosen for the big change in the abundance of T-RF 91-93, showed that species of *Bacteroides* , *Prevotella* and unclassified *Rikenellaceae* could be the candidates for these T-RFs. To verify this assumption we extracted sequences belonging to the 10 most abundant taxa that were found in the tested fecal samples and for comparison downloaded sequences of the type strains of *Bacteroides, Prevotella* and *Rikenellaceae* from RDP. First we located *Msp*I cutting site then calculated the distance to the ENV1 primer binding site, which is the theoretical T-RF size. *Bacteroides*, *Prevotella* and *Barnesiella* yield theoretical sizes ranging from 97-99 nt. This is because before the first *Msp*I recognition site (CCGG), there were two non-consensus nucleotides among the species that belong to the same genus. Type strains belonging to *Rikenellaceae* family produced theoretical T-RF size of 97 nt but the sequences belonging to unclassified members of the *Rikenellaceae* family produced 86-94 nt-long fragments. There were empirically 4-7 nt differences between theoretical size of T-RFs and the values obtained from the T-RF settings used in this study. Thus, with the resolution of T-RFLP, using *Msp*I, it was not possible to distinguish these closely related families of the *Bacteroidales* order. With the same procedure we putatively identified that commonly found T-RF 291-294 represent *Oscillospira* and unclassified *Ruminococcaceae*. T-RF 265 was identified as *Akkermansia* in our previous work and was found in 43 out of 90 participants. Sequences belonging to unclassified *Clostridiales* yield several theoretical T-RF sizes that were difficult to match to the T-RFs generated in this study. T-RF 276 and 277 were putatively identified as *Faecalibacterium* (Fig 8).

Discussion

We hypothesized that consumption of either the tannase producing *L. plantarum* HEAL19 or bilberries fermented by this strain may lead to an anti-hypertensive effect by modulating the microbiota of the GI tract. It had previsouly been reported that a 6 week ingestion of a rosehip drink containing *Lactobacillus plantarum* strain 299v (DSM 9834) was associated with the reduction of various risk markers for cardiovascular diseases such as systolic blood pressure, total cholesterol, leptin and fibrinogen [35]. It was hypothesized in that study that the combination of tannin-rich rosehip and a probiotic with tannase activity resulted in the production

of phenolic acid compounds with anti-inflammatory properties. *Lactobacillus plantarum* strain Heal 19 (DSM 15313) has an even stronger tannase activity as compared to *L. plantarum* 299v. However, in the present study, the tested supplements did not reduce BP and had no apparent effects on either the oral or the fecal microbiota. In another study, the BP lowering effect of blueberry was observed when pre-hypertensive (120/80 mm Hg < BP < 139/89 mm Hg) people with metabolic syndrome consumed a beverage containing 50 g of freeze dried blueberry for 8 weeks [24]. In our study, the dose of fermented bilberry equals to 10 g of fresh bilberries per day. A higher daily dose may be needed to achieve anti-hypertensive effect. In a randomized double-blind crossover study, a 4-week intake of a high level of anthocyanins , extracted from bilberries and black currants, did not lower the high normal BP (BP > 140/90 mm Hg) of otherwise healthy people [36]. The inconsistent findings regarding the anti-hypertensive potential of blueberries could be due to variations among different blueberry species and the consumed amount and the presence of different active compounds in whole blueberries other than pure anthocyanins. Furthermore, the consumption of blueberries may have different effects depending on the pre-hypertensive or hypertensive state of the consumer. Further studies are needed to confirm the anti-hypertensive effect of blueberries. Disappointing findings were also reported in two recent probiotic intervention studies. Intake of *Lactobacillus casei* Shirota for 3 months did not positively affect the metabolic syndrome parameters in adults [37], and intake of *Lactobacillus salivarius* Ls-33 did not confer any beneficial effects on metabolic syndrome in obese adolescents [38] but altered the fecal microbiota composition which, however, could not be linked to metabolic syndrome [39]. Kim et al. used 6 commercial probiotic strains to investigate the response of gut microbiota in healthy adults and found that regardless of the probiotic strain used the intervention did not affect the overall structure of the microbiota [40]. Our results also agree with these findings and support the view of high stability of the fecal microbiota.

Without observing the obvious impact on the microbiota by the study products, we looked into the change in the microbiota through time. T-RFLP analysis showed that changes in relative abundance of certain T-RFs could lead to clustering of the samples. The main driving forces were T-RF 491 and T-RFs 549-550 in the oral microbiota and T-RFs 91-93 in the fecal one. T-RFs 549-550 could be produced from *Streptococcus spp.* (non-published data) and *Bacteroides fragilis* group was found as a one candidate for T-RF 93 in a previous study [41]. It is known that *Streptococcus* is one of the dominant genus in oral microbiota [42] and *Bacteroides spp.* are dominant members of fecal microbiota [43]. These observations led us to question if there are "enterotypes" in the oral and fecal microbiota. The idea of "enterotypes" was proposed in a metagenomic study in 2011, stating that human gut microbiota can be classified into three different enterotypes depending on the dominant level of genera *Bacteroides, Prevotella* and *Ruminococcus* [44]. One built-in limitation of T-RFLP is the difficulty in direct identification of the T-RFs. To identify those T-RFs we applied a high throughput 454 pyrosequencing technique. Due to the currently relatively high cost of the method and the higher observed stability in fecal microbiota, we focused on resolving the fecal microbiota structure in selected individuals that had big changes in T-RFs 91-93. Matching the pyrosequencing result with the T-RFLP profile revealed that the T-RFs 91-93 could represent the genera *Bacteroides, Prevotella, Barnesiella* and unclassified *Rikenellacea*. Among those four genera, *Bacteroides* and unclassified *Rikenellacea* were present in all fecal samples over 3 months, but *Prevotella* and *Barnesiella* only were found abundant in one subject each, no.2 and no.4 respectively (Fig 7). However, due to the lower resolution of T-RFLP it was not possible to distinguish the closely related families belonging to *Bacteroidales* when using the restriction enzyme *Msp*I. In other words, sequences from the four different families may produce the same T-RF in the range of 91-93. However, these T-RFs that were driving the clustering not necessarily were represented by the same genera or family in all samples (Fig 2 and 8). *Ruminococcus* was detected in all the fecal samples at both time points but with low abundance. Thus in the present study no distinct enterotypes could be seen in the fecal microbiota. In other words, the clustering patterns we saw in the PCA analysis of the T-RFLP data do not represent the suggested enterotypes [44]. Instead, our results were pointing that *Bacteroides* and unclassified *Rikenellaceae* were the most dynamic dominant members of fecal microbiota in the hypertensive participants during 3 months.

High level of *Bacteroides* has been associated with long-term diet high in animal protein and fat, while *Prevotella* was linked to diet rich in carbohydrates [45]. The results from pyrosequencing in the present study showed that *Bacteroides* was the most abundant genus, which could be an indication of a high meat intake among the hypertensive individuals. The second dominant bacteria found in this study belonged to unclassified *Rikenellaceae*. *Rikenellaceae* family was found as a dominant member in the fecal microbiota of healthy people [46, 47]. *Rikenellaceae* was one of the dominant families that involved in the carbohydrate metabolism, energy production and synthesis of cellular components [46]. Additionally, *Akkermansia* was absent or less than 0.1% in two subjects but detected at different levels (5-40%) in the other subjects. Similar result was obtained from T-RFLP analysis. *Akkermansia* represented by T-RF265 was detected in 43 out of 90 individuals and showed large variation between individuals regarding change in the abundance by time. These observations suggest that the presence of *Akkermansia* is individual-specific.

One interesting observation, which gives a hint of crosstalk between gut microbiota and host, was that the sharp increase in BP in one subject was accompanied by a dramatic change in fecal microbiota with an increase of *Bacteroidetes* (Fig 4 and 6). Further studies are needed to clarify whether there is a correlation or just a coincidence.

Although the mouth swabs samples were not analyzed by pyrosequencing in this study, comparison of the T-RFLP profiles between the oral and fecal microbiota showed that there was no clear correlation between the two and that the oral microbiota fluctuated more over time. The most fluctuating T-RFs 549-550 could be produced from *Streptococcus spp.* but other closely related taxa may also produce the same T-RFs. Further studies are needed to identify what bacteria are represented by the T-RF 491 and T-RFs 549-550.

In summary, the supplementation of *L.plantarum* HEAL19 alone or with fermented bilberry did not affect the blood pressure in hypertensive people. The supplementation affected neither the diversity nor composition of the oral and the fecal microbiota. The inter-individual variation was larger than intra-individual variation over a 3-months period and no distinct enterotypes could be identified. The overall structure of the oral and fecal microbiota was stable over 3 months but the change in oral microbiota was more dynamic than in fecal microbiota. *Bacteroides* and unclassified *Rikenellaceae* were found to be the two most fluctuating major taxa in the fecal microbiota that was otherwise relatively stable.

Conflict of interest

Bengt Jeppsson and Göran Molin are minority shareholders in the public company Probi AB that coordinated the study and provided the supplements.

Aknowledgements

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Table 1. Characteristic of the six subjects selected for pyrosequencing.

Subject Number	Group	Delta SBP (mm Hg)	Delta DBP (mm Hg)	Delta T-RF 91 (%)	Delta T-RF 92 (%)	Delta T-RF 93-94 (%)
1	P	-35	-11	3.9	0	-24.3
2	P	16	14	-12.9	-4.6	-13.7
3	BL	-12	-10	54.6	0	5.5
4	BL	22	9	0	-12.4	-22.3
5	L	49	20	34.4	0	1.3
6	L	-40	-28	-4.6	36.3	-37.7

Table 2. Characteristic of the participants enrolled in the study.

	P	BL	L
Total	47	47	48
Age (years)	66 (7)	67 (7)	65 (7)
BMI(SD)	27.8 (3.6)	28.8 (4.4)	27.5 (3.9)
Medicating for hypertension	28	30	19
Not medicating for hypertension	19	17	29
Mean SBP (SD)	153.8 (16.6)	151.3 (11.7)	151.5 (13.5)
Mean DBP (SD)	91 (11.7)	91.3 (9.3)	93.4 (8.9)

Table 3. Comparison of diversity calculated from T-RFLP data between the treatment groups before and after consumption of the study products.

	P		BL		L	
	Before	After	Before	After	Median	After
Oral microbiota						
Number of taxa (T-RFs)	11.5±0.6	10.4±0.6	12.7±0.8	11±0.7	12.7±1.1	12.5±1.3
Shannon's diversity index (H)	1.70±0.08	1.59±0.07	1.75±0.08	1.67±0.08	1.77±0.06	1.72±0.08
Simpson's diversity index (1-D)	0.72±0.03	0.70±0.03	0.73±0.03	0.71±0.02	0.74±0.02	0.72±0.02
Fecal microbiota						
Number of taxa (T-RFs)	12.6±1.0	14.0±1.5	14.2±1.4	12.3±0.9	15.2±1.7	12.0±1.1
Shannon's diversity index (H)	1.92±0.11	2.05±0.11	2.11±0.10	1.99±0.08	2.05±0.11	1.92±0.10
Simpson's diversity index (1-D)	0.75±0.04	0.79±0.02	0.81±0.02	0.79±0.02	0.79±0.03	0.77±0.02

P (n=30): people received placebo; BL (n=28): people consumed *L.plantarum* HEAL19 with fermented bilberries; L (n=32): people consumed *L.plantarum* HEAL19. Before: samples taken at the start of the study; After: samples taken after consumption of the studied products for 3 months . Data are mean±S.E.M

Table 4. Comparison of diversity calculated from T-RFLP data between the two sampling timepoints for all participants.

	Before	After
Oral microbiota		
Number of taxa (T-RFs)	12.3±0.5	11.3±0.5
Shannon's diversity index (H)	1.74±0.04	1.66±0.04
Simpson's diversity index (1-D)	0.73±0.01	0.71±0.01
Fecal microbiota		
Number of taxa (T-RFs)	14.0±0.8	12.7±0.7
Shannon's diversity index (H)	2.03±0.06	1.98±0.06
Simpson's diversity index (1-D)	0.78±0.02	0.78±0.01

Before (n=90): samples taken at the start of the study; After (n=90): samples taken after 3 months. Data are mean±S.E.M

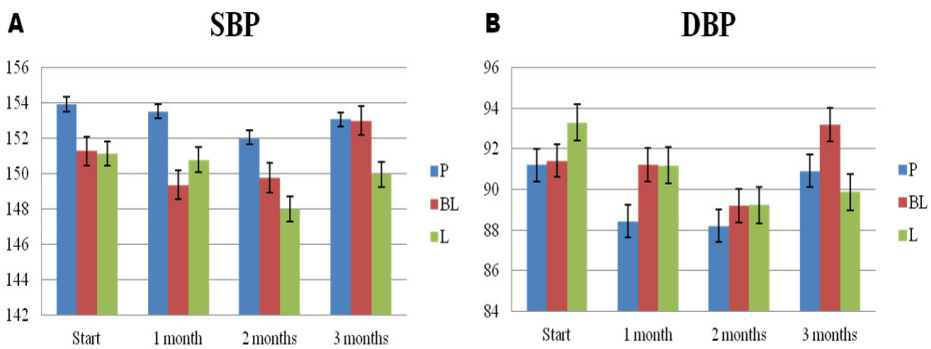

Figure 1. Blood pressure changes over 3 months. (A) Changes in SBP. (B) Changes in DBP. P: placebo group (n=44); BL: group consumed *L.pantarum* HEAL19 with the fermented bilberries; L: group consumed *L.pantarum* HEAL19. Data are means±S.E.M

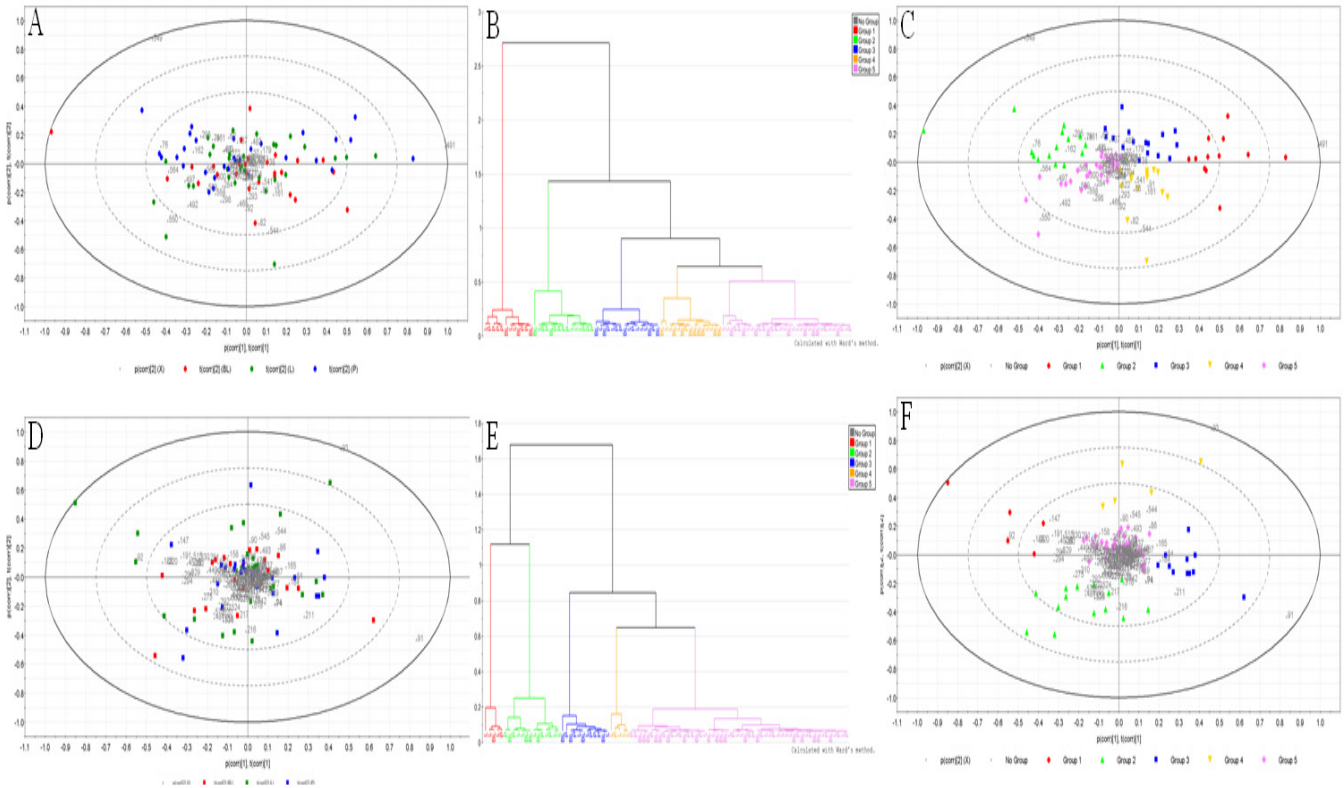

Figure 2. PCA loadings' biplot and dendrogram clustering based on the T-RFLP data. The data matrix was composed of the changes in the abudance of T-RFs over three months. A: Change in the oral microbiota, colored by treatment groups. B. dendogram clustering of the change in the oral microbiota using Ward's method. C. PCA loading's biplot, colored by the dendogram clusters found in B. D: Change in the fecal microbiota, colored by treatment groups. E. dendogram clustering of the change in the fecal microbiota using Ward's method. F. PCA loading's biplot, colored by the dendogram clusters found in E.

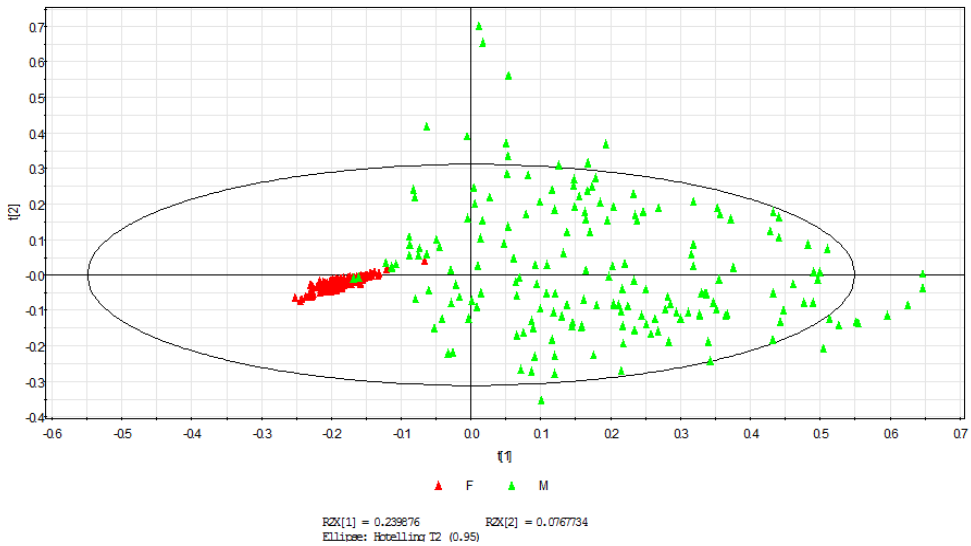

Figure 3. PCA score plot of the fecal and oral microbiota based on the T-RFLP data. Red: Fecal samples; Green: Oral swab samples.

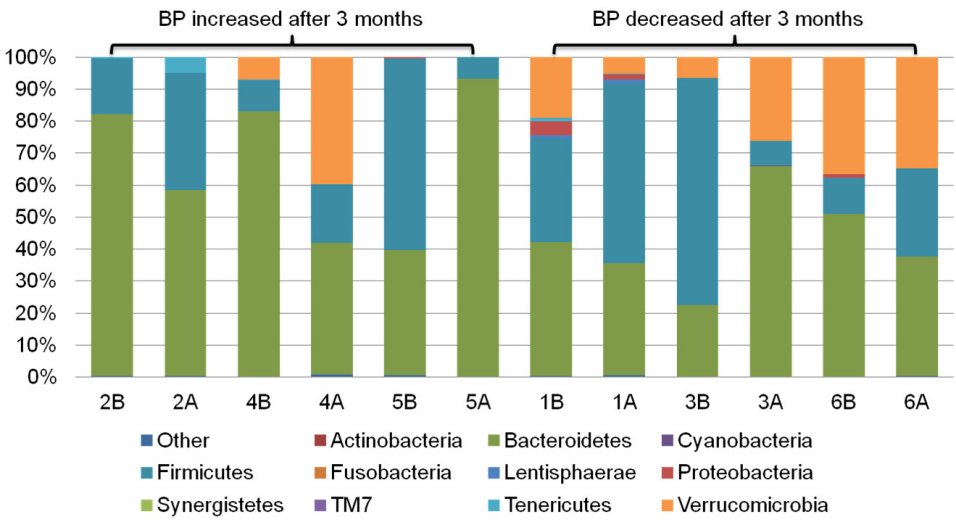

Figure 4. Comparisons of the fecal microbiota composition based on the pyrosequencing data over 3months. Number 1-6 represent tested subjects; B indicates the sample taken at the beginning of the study and A indicates the sample taken after 3 months.

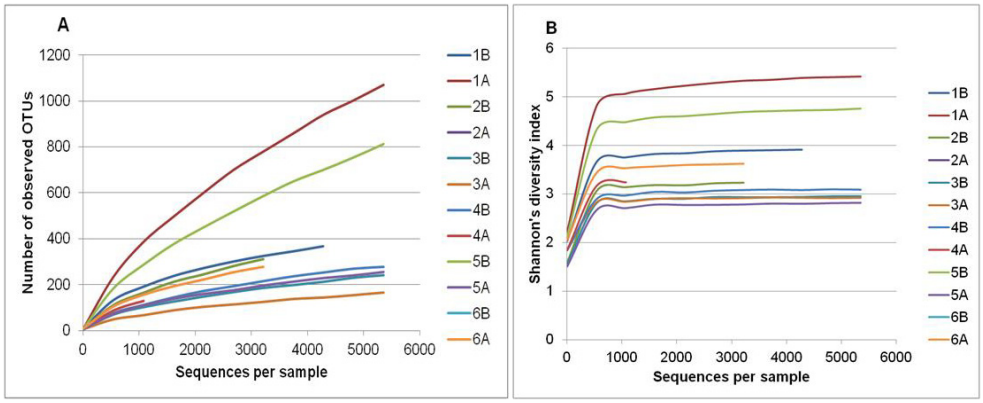

Figure 5. Rarefaction curve analysis. (A) Number of bacterial taxa found in the fecal samples. (B) Shannon's diversity index.

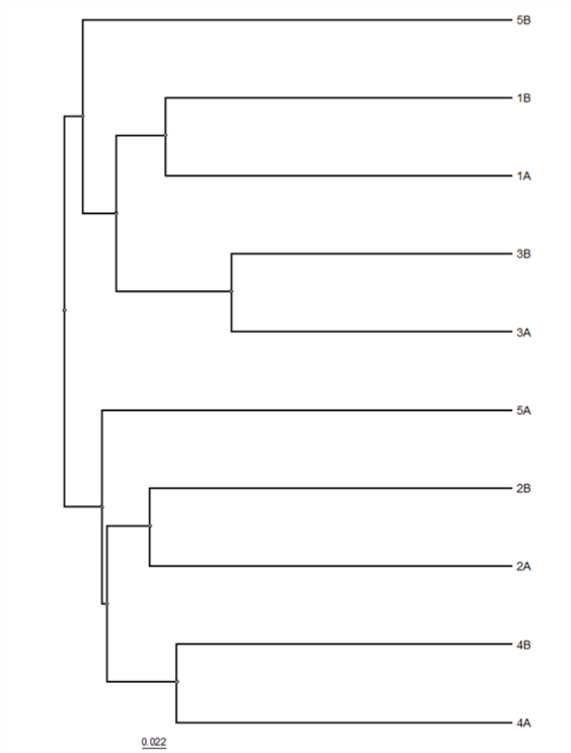

Figure 6. UPGMA clustering (based on pyrosequencing data) of the 10 fecal samples collected from 5 subjects at the beginning of the study and 3 months later. Number 1-5 indicates 5 tested subjects. B indicates the sample taken at the beginning of the study and A indicates the sample taken after 3 months.

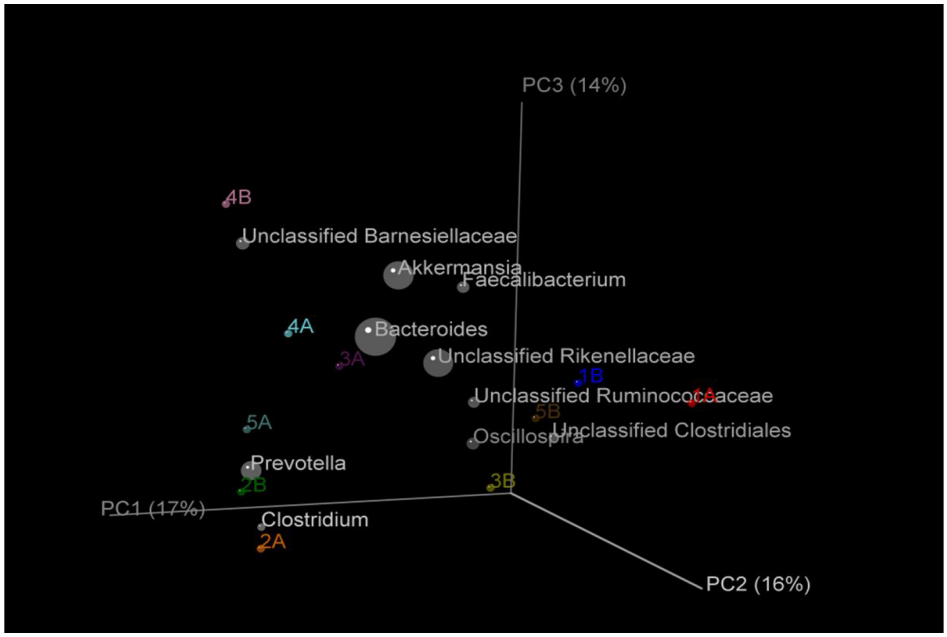

Figure 7. 3D Bi-Plot of the principal coordinate analysis on unweighted UniFrac distance matrix based on pyrosequencing data. The 10 most abundant taxa at genus level were plotted with circles proportional to their abuandance. Number 1-5 indicates 5 tested subjects. B indicates the sample taken at the beginning of the study and A indicates the sample taken after 3 months. Note, *Barnesiellaceae* belonging to the order *Bacteroidales* is not an approved taxonomic name, but an annotated taxa in greenegenes database.

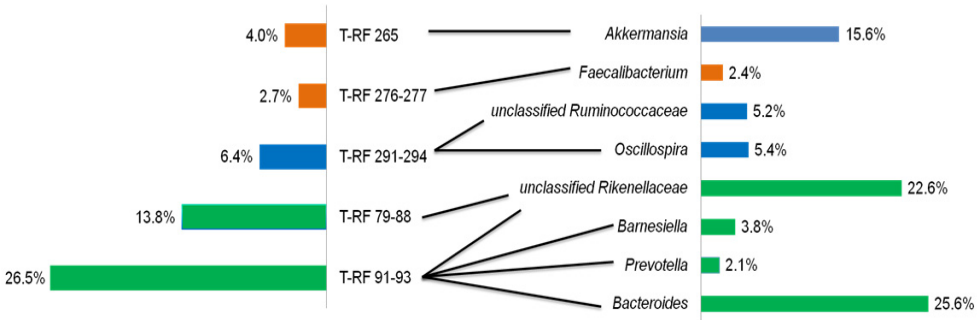

Figure 8. Putative identification of some dominant T-RFs. Left bar chart is generated from T-RFLP data matrix and the average T-RF abundances and the sizes are shown next to the bars. Right bar chart is generated from the pyrosequencing data matrix. The abundance of each taxon is calculated by dividing the number of sequences belonging to the specific taxon with the total number of sequences and is noted next to the bars. Matching between specific T-RFs with and taxonomic group is indicated by lines.

Paper IV

Ileal pouch microbiota of former ulcerative colitis patients without pouchitis

Jie Xu*[1], Anders Falk[2], Crister Olsson[3], Henrik Thorlacius[2], Bengt Jeppson[2], Siv Ahrné[1], Göran Molin[1]

[1]Laboratory of Food Hygiene, Department of Food Technology, Engineering and Nutrition, Lund University, Box 124, SE-221 00 Lund, Sweden

[2]Surgery Research Unit, Department of Clinical Science, Lund University, Skåne University Hospital, 205 02 Malmö, Sweden

[3]Department of Biosystem and Technology, Swedish University of Agricultural Science, P.O. Box 103, 230 53 Alnarp, Sweden

*Corresponding author. Email: Jie.Xu@appliednutrition.lth.se Phone: +46-46-222 98 16

Abstract

Dysbiosis has frequently been observed in patients with ulcerative colitis (UC). The patients with pouchitis have symptoms resembling UC. This implies a role of the microbita in the development of pouchitis. In the present study we followed changes in the pouch mucosal microbiota of two patients at two sampling time points (one month and one year after surgery). The microbiota of the two patients differed a lot and one of them underwent drastic change regarding to the bacterial diversity and composition. Comparison of the microbiota at one year after surgery of fourteen patients showed a large individual variation. *Clostridium, Bacteroides* and genera of *Lachnospiraceae* were the dominant members while *Megamonas* or *Streptococcus* was overrepresented in some patients. *Haemophilus*, *Aggregatibacter*, *Turicibacter* and *Sutterella* were detected with high abundance in some of the patients. The load of *Faecalibacterium prausnitzzi* generally increased by time. Further research is needed to find out if any of the observed differences in the pouch microbiota is linked to a predisposition of pouch mucosal inflammation.

Key words: Ulcerative colitis, pouch, microbiota

Introduction

Development of dysbiosis in the ileal pouch after ileostomy closure has been suggested to be involved in the etiology of pouchitis [1]. Picturing the development of pouch microbiota composition has been done using both culture-dependent and 16S rRNA gene based methods. However, the findings are inconsistent. The pouch microbiota has been reported to develop towards a colon-like microbiota [2, 3], but it has also been said that it develops into a musoca associated microbiota distinctly different from that of a normal one in colon [4]. McLaughlin et al. found that *Proteobacteria* made up a higher proportion than *Bacteroidetes* and *Fimicutes* (two major phyla in colon) in the pouch of former patients with ulcerative colitis (UC), with or without pouchitis [4]. In contrast, dominance of *Fimicutes* has been reported in the stool samples from both patients with normal healthy pouches and those with pouchitis [5]. Members of the "*Clostridium* group", *Enterobacteriaceae* and *Streptococcus* were found to be associated with mucosa of the healthy pouch, whereas members of *Fusobacterium* and the depletion of *Stretopcoccus* species were found to be associated with inflamed mucosa in pouchitis [6]. In another study, *Clostridiaceae* was found in higher amount in the patients with chronic or relapsing pouchitis [7]. It has been reported that *Faecalibacterium prausnitzzi*, with a proposed anti-inflammatory capacity [8], reduced in UC patients [9] and increased in remission but remained low in relapse [10].

In the present work, we aimed to explore the early composition of the pouch microbiota in former UC patients without pouchitis. Terminal restriction fragment length polymorphism (T-RFLP) and 454 amplicon pyrosequencing was used to map the pouch microbiota and SYBR green quantitative PCR (qPCR) was used to quantify *F. prausnitzzi*.

Materials and Methods

Samples. Mucosal biopsy samples were taken from normal pouches of former UC patients. Fourteen patients were included and the samples were taken one month and one year after ileostomy closure. The detailed procedure has been described elsewhere [2].

DNA extraction. Genomic DNA was extracted according to the manufacturers' instruction with EZ1 DNA tissue kit (Qiagen AB, Sollentuna, Sweden) on the EZ1 Advanced XL BioRobot workstation (Qiagen). Details of the procedure were described elsewhere [11].

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis. The 16S rRNA gene was amplified by FAM labeled forward primer ENV1 (5′-FAM-AGAGTTTGATIITGGCTCAG-3′) and an unlabeled reverse primer ENV2 (5′-CGGITACCTTGTTACGACTT-3′). The extracted DNA was diluted 10 to 100 times for each sample and 1 uL of the diluted DNA was used as template in 25 µL of the PCR reaction containing 0.4 µM of FAM-ENV1 primer and 0.2 µM of primer ENV2, 2.5 µL of 10x TopTaq PCR buffer (containing 15 mM $MgCl_2$), 0.2 mM of each dNTP and 1.25 U of TopTaq DNA polymerase (Qiagen). Triplicates reactions were performed for each sample. The PCR conditions were as follows: 94°C for 3min, followed by 30 cycles of 94°C for 30 s, 54°C for 40 s and 72°C for 1 min. An additional extension at 72°C for 10 min was performed at the end of PCR. The amplicons were purified with MinElute PCR purification kit (Qiagen), and 200 ng of the purified DNA of each sample was digested with endonuclease *Msp*I (Fermentas Life Science, Burlington, Canada). The resulting 16S rDNA fragments were analyzed on an ABI 3130xl Genetic analyzer (Applied Biosystems, Foster city, 212 CA, USA) at DNA-lab (SUS, Malmö, Sweden). The T-RFLP data was analyzed with the Genemapper® software version 4.0 (Applied Biosystems) with local southern algorithm. A free software PAST (version 2.17b, http://folk.uio.no/ohammer/past/) was used to calculate Shannon's diversity index using T-RFLP data.

Pyrosequecing of 16S rDNA amplicons (V1-V3). 16S rDNA amplification was only successful for two samples taken from one month after surgery. Thus, fourteen samples taken from fourteen patients one year after surgery and two samples taken from two individuals one month after surgery were chosen for

pyrosequencing. The V1-V3 regions of the 16S rRNA gene was amplified with a forward primer (5′-MIDx-AGAGTTTGATIITGGCTCAG-3′) and a reverse primer (5′-MIDy-GWATTACCGCGGCKGCTG-3') where MID is a unique multiplexing identifier composed of 10 nucleotides that are used for barcoding the sequences. The MIDs are different for the forward and reverse primers. The PCR reactions were prepared same way as described above and 35 cycles were performed. The PCR cycling conditions were 94°C for 3min, followed by 30 cycles of 94°C for 30 s, 54°C for 40 s and 72°C for 1 min. An additional extension at 72°C for 10 min was performed at the end of PCR. The amplicons were purified with MinElute Gel Extraction kit (Qiagen). The concentration of the DNA was measured by Nanodrop ND-1000 (Saveen Werner, Limhamn, Sweden). Amplicons were pooled in equal amount to have a final concentration of 25 ng/µL. The PCR products were subsequently amplified by emulsion PCR and sequenced at MWG, Germany using GS FLX+ chemistry.

Sequence analysis. Sequences were analyzed with an open source software package Quantitative Insights Into Microbial Ecology (QIIME 1.7.0) [12]. Two mapping files were created for forward and reverse reads. Using the split library script the sequences were filtered for sequence length > 200 nt, quality score window >50, having correct primer sequence, maximum length of homopolymer of 6 nt. Reverse primer sequence was trimmed and any subsequence sequence was also removed. The filtered sequences were assigned to samples according to the barcodes. The reverse reads were reverse complemented and then concatenated with forward reads. Operational Taxonomic Units (OTUs) were picked with 97% sequence similarity using UCLUST [13]. Most abundant sequences found in each OTU was picked as representative sequences and aligned against Greengenes core set [14] using PyNAST [15] with a minimum sequence identiy of 75% and a minimum sequence length of 300 nt. Taxonomy was assigned to the representative sequences using Ribosomal Database Project (RDP) Classifier with minimum confidence score of 0.8 [16]. Potential chimeras were identified with ChimeraSlayer [17] and removed from the aligned sequences. An OUT table was built with non-chimeric and non-PyNAST failure sequences and used for downstream analysis.

Quantification of *F. prausnitzzi* using SYBR green qPCR. *Faecalibacterium prausnitzii* DSM 17677 stains was used for constructing plasmid standard. The detailed procedure has been described elsewhere [11]. The *F. prausnitzii* was amplified with a forward primer (5'-CCATGAATTGCCTTCAAAACTGTT-3') and a reverse primer (5'-GAGCCTCAGCGTCAGTTGGT-3') [9]. The PCR reactions were performed using Rotor-Gene SYBR Green PCR kit (Qiagen) in a real-time PCR cycler Rotor-Gene Q (Qiagen). The PCR reaction was prepared in 20 µl consisting 10 µl of 2× Rotor-Gene SYBR Green PCR Master mix, 0.5 µM of each primer and 2 µl of template DNA. Duplicate reactions were performed for each sample, standard and negative controls. The PCR condition was set as following: activation at 95 °C for 5 min followed by 40 cycles with denaturing at 95°C for 5 s followed by annealing and extension at 60°C for 10 s. Melt curve analysis was performed for each run to check the specificity of the primers.

Results

T-RFLP. The 16S rRNA gene amplification was not successful for the samples taken one month after surgery, probably due to too low levels of the 16S rRNA genes at this early stage. Thus, only data from samples taken one year after surgery were analyzed with T-RFLP. In total 43 T-RFs were detected, and there was a large individual difference in the number of detected T-RFs (min to max, 3 to 19) and in Shannon's diversity indices (0.5 to 2.5).

Ileal pouch mucosa associated microbiota composition revealed by pyrosequencing. The sequences were clustered into Operational Taxonomic Units (OTUs) with 97% similarity, and in total 125739 non-chimeric sequences were obtained. At phylum level *Firmicutes* (92%) was predominant followed by *Bacteroides* (4%) and *Proteobacteria* (3%). *Fusobacteria, Actinobacteria, Verrucomicrobia*, candidate phylum TM7 and sequences that could not be assigned to any phylum made up the rest 1% (Fig. 1).

Bacterial compositions at genus level in all patients were illustrated in figure 2. Only taxa that had more than 0.5% abundance were plotted. For patient 1 and 2, samples taken at both sampling time points were analyzed and compared. For patients 3-14, samples taken one year after surgery were analyzed.

Patient 1. The diversity of the microbiota was higher one year after surgery when compared to one month after operation (Fig. 3). The largest change in the microbiota composition between the two sampling time points was that *Clostridium* increased dramatically one year after surgery, from 0.5% to 20.5%. *Coprococcus* increased around 7 fold (1month vs. 1year, 1.4% vs. 9.4%), *Blautia* increased around 6 fold (1month vs. 1year, 2.5% vs.15.6%) and *Faecalibacterium* increased around 2 fold (1 month vs. 1 year, 4.5% vs. 9.7%). The increase was also seen in the less abundant bacteria. *Dorea* made up 0.4% at one month after surgery and increased to 2 % one year after surgery. *Pediococcus* was not detected one month after surgery but found at one year after surgery with abundance of 1.1%. On the other hand, the most abundant genus *Lachnospira* showed a sharp drop from 29.7% at one month to 1.1% after one year. However, other genera belonging to *Lachnospiraceae* family increased one year after surgery. *Roseburia*, *Sutterella* and *Lactobacillus* had also decreased after one year. *Halomonas* (2.2%) and *Veillonella* (1.1%) were found to be less than 0.5% one year after surgery.

Patient 2. The microbiota diversity dramatically increased one year after surgery when compared to one month after surgery (Fig. 3). After one month, the family *Peptostreptococcaceae* was predominant (87.9%) and genera *Turicibacter* and *Clostridium* made up 9.8% and 1% of the microbiota, respectively. After one year, *Bacteroides, Blautia, Haemophilus* and *Lachnospira* were present with similar abundance (16.6%~23.5%) and *Faecalibacterium*, *Aggregatibacter*, *Streptococcus* and *Roseburia* were found between 0.5% and 3.7%. *Turicibacter* and *Clostridium* decreased till below 0.5% after one year.

Patient 3. In this patient, *Megamonas* was dominant (43.9%). *Bacteroides, Blautia* and *Roseburia* were found with similar abundance (10.2%~14.4%). *Coprococcus* made up 3.3% and *Dorea, Sutterella* and *Faecalibacterium* were found between 0.5% and 0.7%. In addition, bacterial sequences that could not be assigned to genus level from different higher taxa such as *Lachnospiraceae* (6%), *Bacteroidales* (5%) and other taxa that composed 12.6% of the total number of sequences.

Patient 4. In this patient, *Clostridium* was dominant (77.1%). *Turicibacter, Bacteroides* and *Blautia* made up 15.6%, 1.5% and 1.3% respectively. Bacterial sequences that could not be assigned to genus level from different higher taxa composed 3% of the total sequences.

Patient 5. In this patient, *Blautia* was dominant (39.5%). Other abundant genera were *Faecalibacterium* (10.1%), *Roseburia* (9.5%), *Bacteroides* (7.2%), *Turicibacter* (6.2%), *Coprococcus* (3.8%), *Clostridium* (1.6%), *Lachnospira* (1.4%) and *Bifidobacteria* (0.6%). In addition, bacterial sequences that could not be assigned to genus level from different higher taxa such as *Lachnospiraceae* (12%), *Peptostreptococcaceae* (5%) and other taxa that composed 19.7% of the total sequences.

Patient 6. In this patient, unclassified *Lachnospiraceae* was dominant (41.2%). The other abundant genera were *Faecalibacterium* (18.3%), *Blautia* (12.4%), *Coprococcus* (5.4%), *Lachnospira* (4.9%), *Roseburia* (3.4%), *Dorea* (2.1%), *Sutterella* (0.9%) and *Ruminococcus* (0.8%).

Patient 7. This patient had similar pouch microbiota as patient 6. Unclassified *Lachnospiraceae* (35.1%) was the most abundant taxum. Other abundant genera were *Blautia* (22.3%), *Faecalibacterium* (20%), *Coprococcus* (6.6%), *Lachnospira* (3.5%), *Roseburia* (2%), *Dorea* (1.3%), *Turicibacter* (0.7%) and *Sutterella* (0.6%).

Patient 8. In this patient, *Clostridium* was dominant (55.9%). Bacterial sequences that could not be assigned to genus level from different higher taxa such as *Peptostreptococcaceae* (7%), *Streptococcaceae* (4%), *Lachnospiraceae* (3%) and other taxa that made up 19.8% of the total sequences. Other abundant genera were *Prevotella* (5.2%), *Veillonella* (4.3%), *Streptococcus* (3.8%), *Faecalibacterium* (3.5%), *Lachnospira* (2.7%), *Bacteroides* (1.6%), *Coprococcus* (1.4%) and *Haemophilus* (0.9%).

Patient 9. In this patient, *Bacteroides* was dominant (37.7%). Bacterial sequences that only could be assigned to taxa above the genus level were, for example, *Lachospiraceae* (14%), *Peptostreptococcaceae* (4%), Ruminococcaceae (4%) and "other taxa" that made up 25.3% of the total sequences. Other abundant genera than *Bacteroides* were *Faecalibacterium* (10.7%), *Roseburia* (9.2%), *Blautia* (5.3%), *Lachnospira* (3.4%), *Haemophilus* (1.8%), *Coprococcus* (1.6%), *Clostridium* (1.3%), *Ruminococcus* (1%) and *Streptococcus* (0.6%).

Patient 10. In this patient, unclassified *Lachnospiraceae* was dominant (35.4%). *Roseburia* and *Faecalibacterium* were detected in similar level (17.4% and 17.2% respectively). Bacterial sequences that only could be assigned to taxa above the genus level were for example *Clostridia* that made up 10% of the total sequences. Other abundant genera than *Roseburia* and *Faecalibacterium* were *Lachnospira* (6.9%), *Coprococcus* (2.5%), *Blautia* (1.7%), *Clostridium* (1.7%), *Halomonas*(0.6%), *Dorea* (0.6%) and *Lachnobacterium* (0.5%).

Patient 11. In this patient, *Streptococcus* was dominant (33.2%). Bacterial sequences that only could be assigned to taxa above genus level belonged for example to *Lachnospiraceae* (15%), *Enterobacteriaceae* (3%) and "other taxa" which made up 23.4% of the total sequences. Other abundant genera besides *Streptococcus* were *Blautia* (10.5%), *Bacteroides* (9.1%), *Roseburia* (8.9%), *Faecalibacterium* (6.7%), *Coprococcus* (2.1%), *Clostridium* (1.9%), *Prevotella* (1.4%), *Escherichia* (0.7%) and *Veillonella* (0.6%).

Patient 12, 13 and 14. The number of sequences obtained from these three patients were less than 500, thus they were excluded from principal coordinate analysis (PCoA) on UniFrac distance matrix. The composition of the microbiota in these patients are shown in figure 1 and 2, however, due to the low number of reads the identification results should be interpreted with caution.

The ten most abundant taxa at genus level found among the eleven patients (Patient 12, 13 and 14 were excluded due to the low number of sequences) at both sampling time points were *Clostridium, Blautia, Roseburia, Lachnospira,* unclassified *Lachnospiraceae*, *Bacteroides, Faecalibacterium,* unclassified *Petostreptococcaceae* and *Megamonas* (Fig. 4).

Putative identification of T-RFs. A large individual difference was observed when comparing the T-RFLP profile of each sample. However, some dominant T-RFs were shared by several patients, which resembled the pyrosequencing result. Putative identification was done by comparing each T-RFLP profile with pyrosequencing result. Sequences of suspected taxa were extracted from the pyrosequencing reads and the sequences of corresponding type species were downloaded from RDP and cut with *Msp*I *in silico* to obtain theoretical sizes of the T-RFs of interest. Empirically 4-7 nt differences exist between theoretical size of T-RFs and the values obtained from experiment settings used in this study. The putative identification of the most abundant T-RFs detected in the samples is shown in table 1.

Quantification of Faecalibacterium prausnitzii. The amount of *F.prausnitzii* varied between individuals and the largest difference was around 100 fold (Table 2). In samples taken one month after surgery, only patient no. 1 had sufficient amount of *F.prausnitzii* to fall within the detection range. In patients 13 and 14, *F.prausnitzii* was not detected at any of the time points.

Discussion

We have used T-RFLP and pyrosequencing to analyze the pouch mucosa associated microbiota. The T-RFLP profile showed a large individual variation, but some T-RFs with high abundance were detected commonly among the patients. By comparing the T-RFLP profile with the results from pyrosequencing we could putatively identify those major taxa. Although with lower resolution, T-RFLP pattern were in accordance with the pyrosequencing results. Thus, T-RFLP could be used as a first step to screen the microbiota structure.

Change in the pouch microbiota of two of the patients with time. The pouch mucosa associated microbiota in patient 1 and patient 2 were different at the first sampling time point (one month after surgery) and developed into more diverse microbiota after one year. Patient 1 did not have a dramatic change regarding to

the composition or the overall diversity over one year. However, rearrangement of dominant bacterial genera was observed and notably the increase of *Clostridium* and several genera of *Lachnospiraceae* (other than *Lachnospira*) and a decrease in *Sutterella* (class *ß-proteobacteria*). *Lachnospiracea* has commonly been found in the stool samples from patients with normal pouches, chronic pouchitis or familial adenomatous polyposis pouches with highest level in normal pouch stools [5]. Patient 2 had a much more simplified microbiota compared to patient 1 at one month after surgery, characterized by the predominance of *Peptostreptococcaceae* (other than *Peptostreptococcus*) and high abundance of *Turicibacter*. *Petostreptococcaceae* is a dominant family of Gram positive anaerobes in the human intestinal microbiota [18]. In patients with colorectal cancer, increased level of mucosa associated *Peptostreptococcaceae* has been reported [19]. Interestingly, at one year after surgery bacteria belonging to this family were no longer detected. *Turicibacter* is a genus of Gram positive strict anaerobes belonging to *Erysipelotrichaceae* (phylum *Firmicutes*) [20], and has been found dominant at the time of pouch construction in one patient in a previous study [2]. At one year after surgery, the pouch microbiota of patient 2 became much more diverse. One interesting observation was the high abundance of *Haemophilus* and *Aggregatibacter* (family *Pasteurellaceae*, class *γ-proteobacteria*) in patient 2. *Haemophilus* has been overrepresented in the stool samples of children with UC in comparison to the CD patients or non-IBD controls [21]. *Aggregatibacter* has been found to be abundant in the salivary microbiota of healthy individuals [22], however, some species such as *Aggregatibacter actinomycetemcomitans* can cause oral infections [23]. If the sudden change in the pouch microbiota composition and the presence of these two potentially harmful genera are linked to a predisposition of pouch inflammation needs to be further investigated.

Pouch microbiota at one year after surgery. Although a large individual variation has been observed, the profile of the dominant genera showed some similarity between the patients. *Clostridum, Bacteroides* and genera of *Lachnospiraceae* were often found to be the most abundant members, while one patient had *Megamonas* (family *Veillonellaceae*) and another patient had *Streptococcus* as the most abundant genus. Clostrida clusters XIVa and IV (family *Lachnospiraceae* and *Ruminococcaceae*), *Bacteroides* and *Enterobacteriaceae* have been found dominant in an earlier study which was performed on two patients [2]. However, in the present study, *Enterobacteriaceae* was found abundant only in patient 11 (3%) and in patient 14 (7%). This difference between studies could rise from the individual difference, larger number of patients in the present study and also the sequencing methods. *Megamonas* species have been isolated from feces of healthy humans [24], and have not been reported to associated with pouchitis or UC. *Megamonas* species have been found at significantly higher levels in stool samples from healthy individuals when compared to the patients with colon cancer [25]. *Streptococcus* was found to be associated with healthy pouch mucosa but was absent in the inflamed pouch [6]. In addition, *Streptococcus* has been found in high abundance in the normal mouth and jejuna microbiota [26, 27].

Faecalibacterium and several genera of *Lachnospiraceae* including *Lachnospira*, *Blautia*, *Roseburia*, *Coprococcus*, *Dorea* and *Lachnobacterium* were commonly found abundant among the patients. Bacteria belonging to these taxa have been commonly associated with healthy colon mucosa [19, 28]. Thus, the pouch microbiota observed in this study indicates the development of a colon-like microbiota. Almeida et al. using culturing method found that *Veillonella* species were the most prevalent bacteria in the ileum and colon of healthy individuals as well as in patients with UC. Moreover, higher prevalence of *Veillonella* species was found at earlier time points after ileostomy closure (2 months vs. 10 months) [29]. In the present study, the genus *Veillonella* was detected in some patients but not as predominant bacteria. The lower amount seen here could be due to the cultivation-independent methods used in this study. *Turicibacter* was found abundant in three patients even at one year after surgery. *Turicibacter* has been isolated from a blood culture from a patient with acute appendicitis [30], but also from feces of a healthy individual [31]. Unfortunately, not much is known about the ecological role of this genus. The prevalence of *Sutterella* was 57.1% (8 out of 14 patients with abundance ranging from 0.2% to 1.7%) at one year after surgery. The type species *Sutterella wadsworthensis* has been isolated from infected gastrointestinal tract in humans and found to be resistant to bile [32] , but recent studies suggested that it is more likely a member of intestinal commensals [33, 34]. On the other hand,

in both of these studies it was found abundant in connection to diseased individuals. Furthermore, Walker et al. analyzed inflamed and non-inflamed colonic mucosa associated microbiota and observed that *Sutterella* significantly decreased in the inflamed tissue in one out of six patients with Crohn's disease, no change was found in patient with UC [35] . *Sutterella* has also been found abundant in the fecal microbiota of children with autism spectrum disorder [36] and in feces of dogs with acute hemorrhagic diarrhea [37]. In addition, *Sutterella* has been associated with inflammatory outcome in the pouch created by ileal pouch-anal anastomosis [38].

In conclusion, a large individual variation exists in the pouch mucosal microbiota. One year after the construction of the pouch, the microbiota was commonly dominated by *Clostridium, Bacteroides* and different genera of *Lachnospiraceae*. However, predominance of other genus such as *Megamonas* or *Streptococcus* was also observed. In addition, *Haemophilus*, *Aggregatibacter, Turicibacter* and *Sutterella* were detected in high abundance in some patients. The amount of *F. prausnitzzi* generally increased with time, but the level differed dramatically among the patients. Even if there was a large variation in the pouch microbiota, none of the patients have shown any symptoms or signs of pouchitis one year after surgery. Further studies on finding whether the drastic change in the microbiota or the certain bacterial species are linked to a predisposition of pouch mucosal inflammation will be performed.

Conflict of Interests

The authors declare no conflict of interests.

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Table 1. Putative identification of the major T-RFs.

T-RF size (relative abundance)*	Putative taxa (relative abundance)#
T-RF 90 and T-RF 93 (6.1%)	*Bacteroides* (10%)
T-RF 146 (0.7%)	*Turicibacter* (2%)
T-RF 217 and T-RF 219 (21.3%)	*Lachnospireaceae (34%)*
T-RF 275 and T-RF 277 (6.4%)	*Faecalibacterium* (8%)
T-RF 289 (5.4%)	*Megamonas (3%)*
T-RF 488 and T-RF 490 (9.4%)	*Enterobacteriacea, Haemophilus, Sutterella* (2%)
T-RF 515 (10.4%)	*Clostridium (13%)*
T-RF 550 (14.1%)	*Streptococcus (9%)*

*Calculated based on the relative peak area of the detected T-RFs.
Calculated by dividing the number of sequences assigned to the corresponding taxa by the total number of sequences.

Table 2. Quantification of *Faecalibacterium prausnitzii* using SYBR green qPCR.

Patients	*Faecalibacterium prausnitzii* (log copies/g)
1B	8.8
1	9.8
2B	ND
2	8.7
3	8.0
4	7.2
5	8.2
6	9.5
7	9.1
8	8.4
9	9.2
10	8.0
11	9.1
12	10.2
13	ND
14	ND

ND: not detected

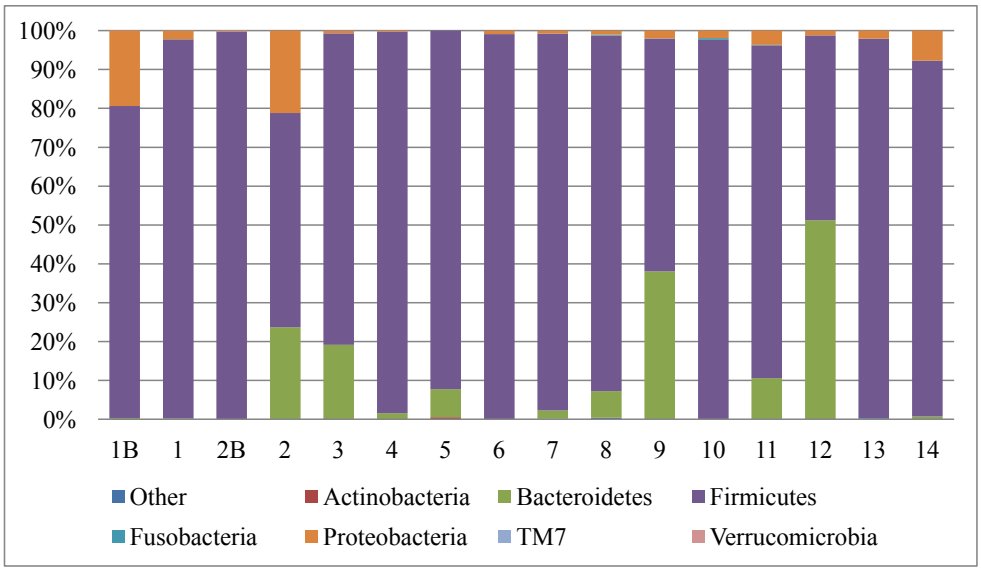
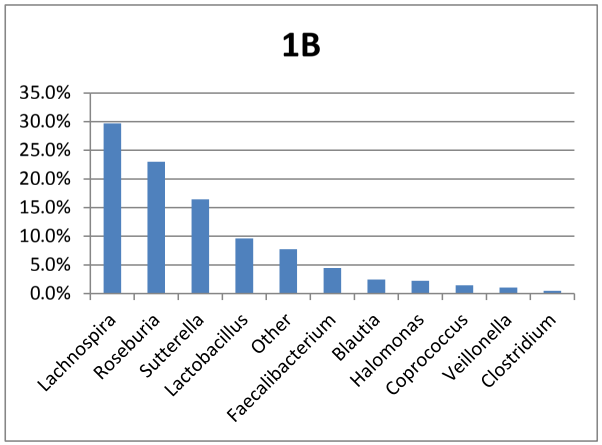
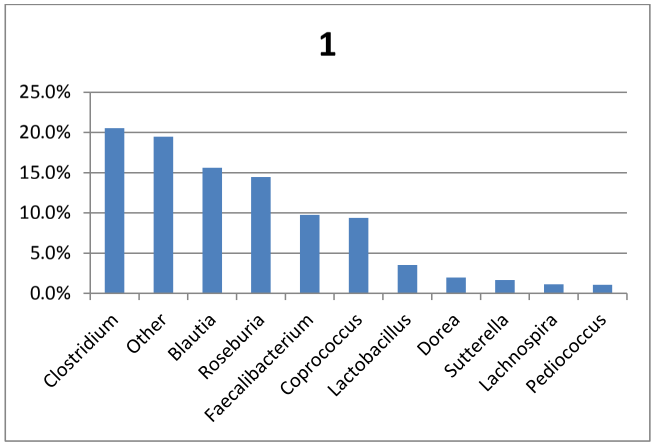
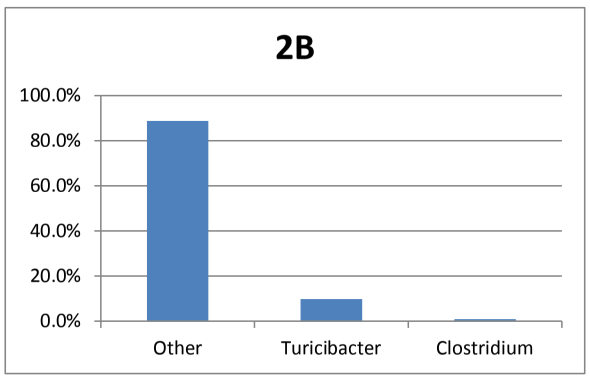


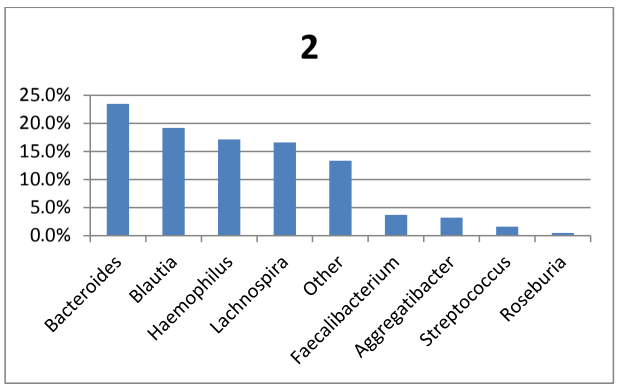
Figure 1. ileal pouch microbiota composition at phylum level. Patients were numbered 1-14. B denotes sample taken one month after surgery.



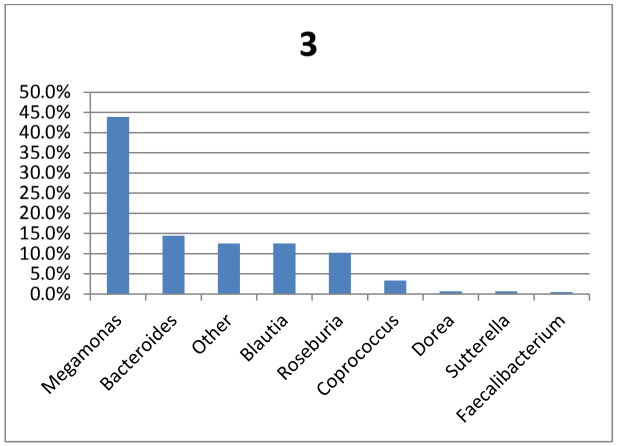


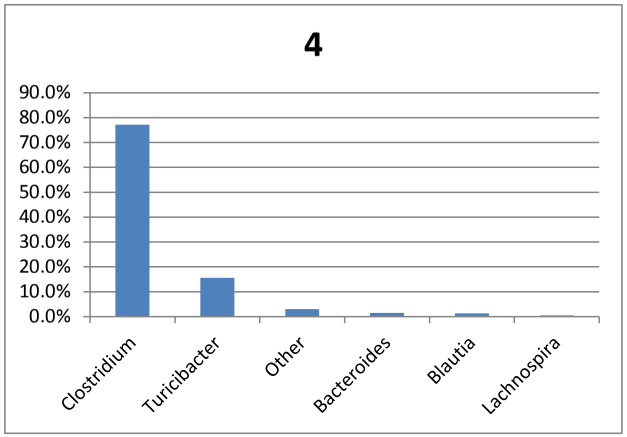
Note: in 1, 15% sequences of "Other" were from *Lachnospiraceae*

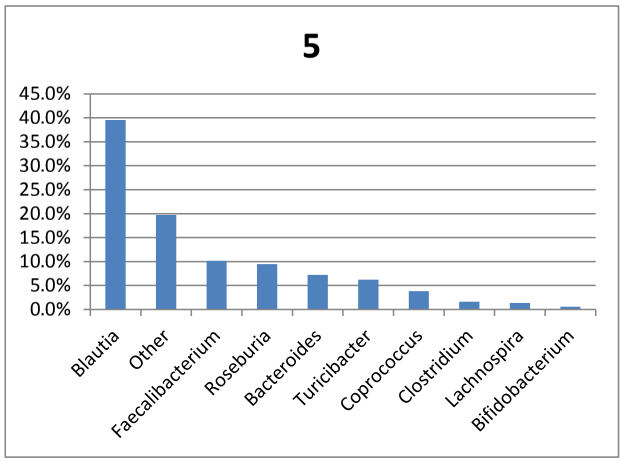


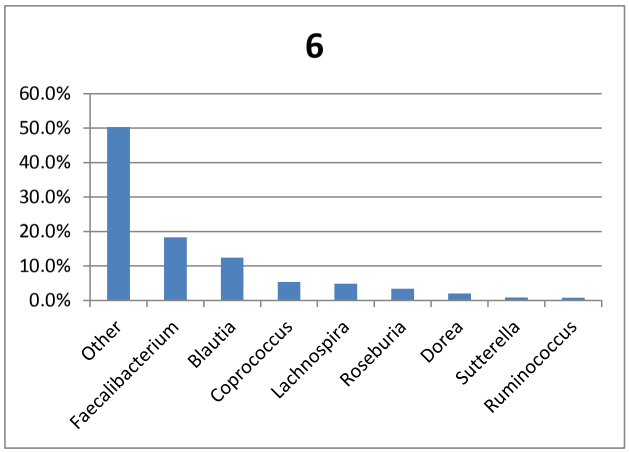


Note: In 2B, 87.9% of "Other" sequences were belong to *Peptostreptococcaceae*

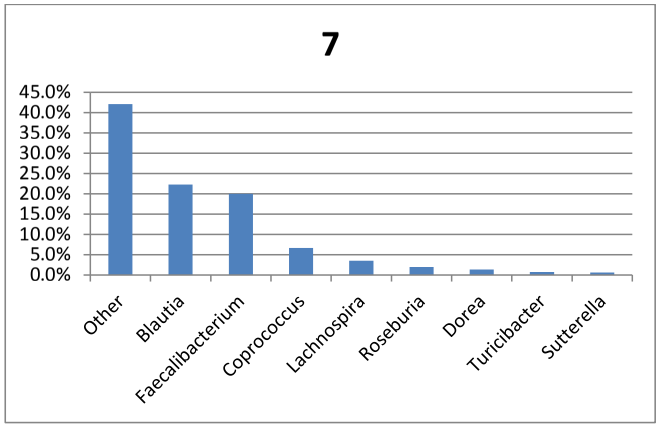


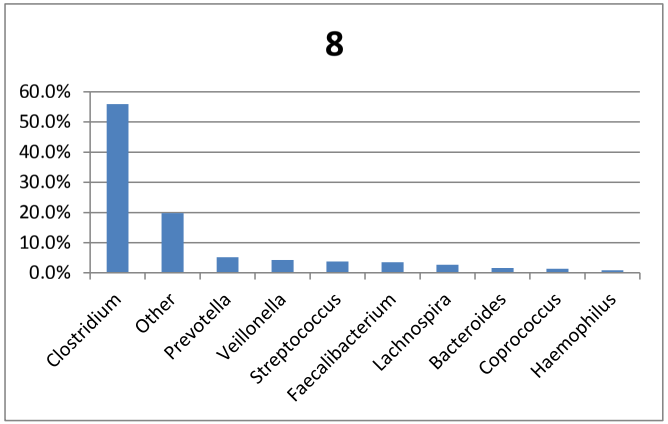




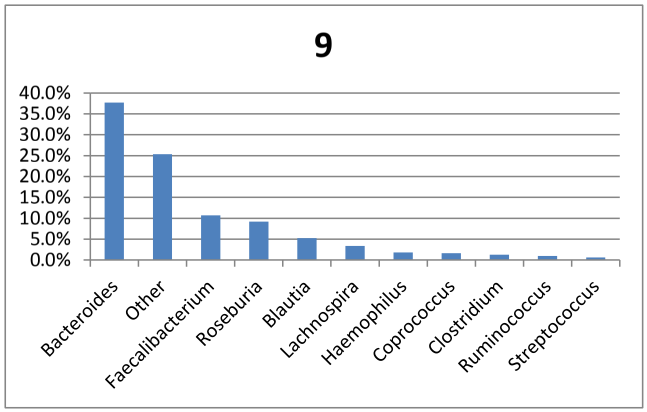


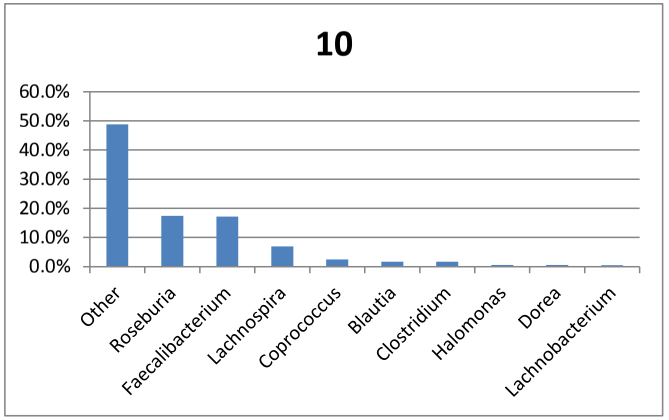
Note: In 6, 41.2% of the sequences of "Other" were from *Lachnospiraceae*



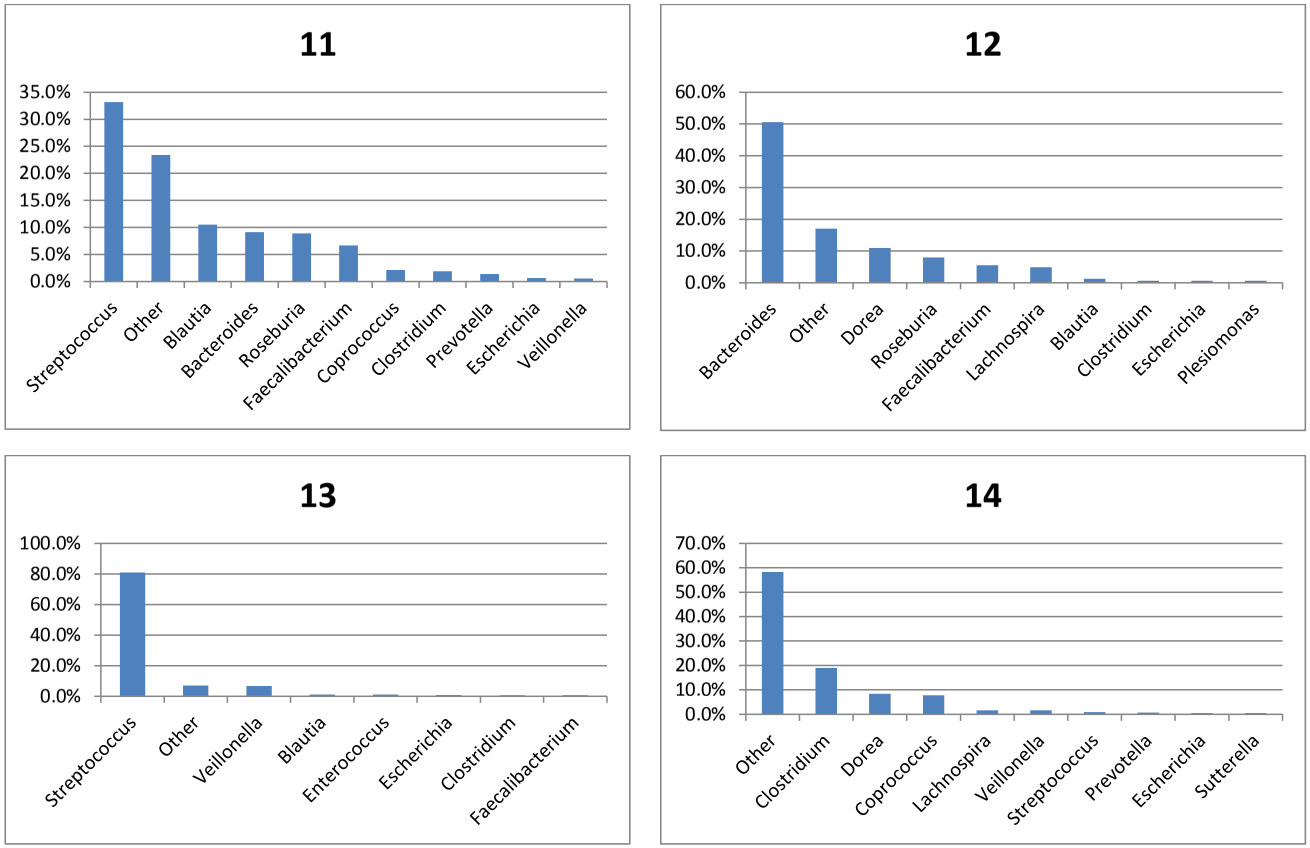


Note: In 7, 35.1% of sequences of "Other" were from *Lachnospiraceae*





Note: In 10, 35.4% of sequences of "Other" were from *Lachnospiraceae*



Note: In 14, 50.1% of "Other" sequences were from *Lachnospiraceae*

Figure 2. Ileal pouch microbiota composition in all patients at genus level. Only taxa with abundance higher than 0.5% were shown.

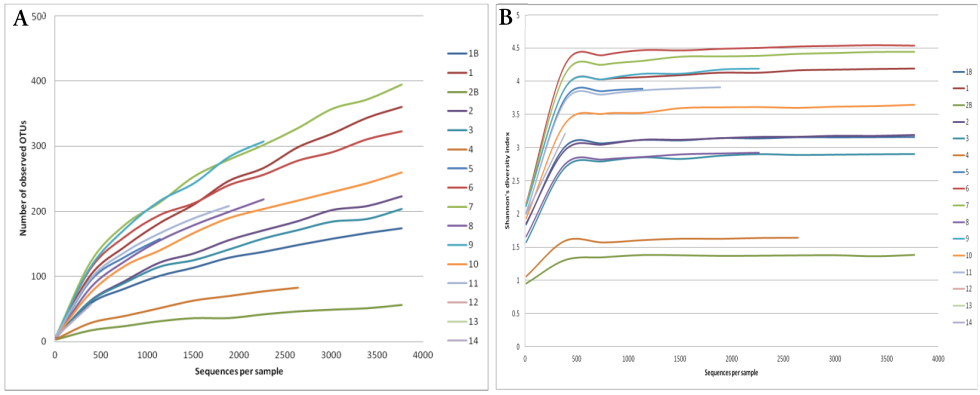


Figure 3. Rarefaction curve analysis. (A) Number of bacterial taxa found in the ileal pouch. (B) Shannon's diversity index. Patients were numbered 1-14. B denotes sample taken one month after surgery.

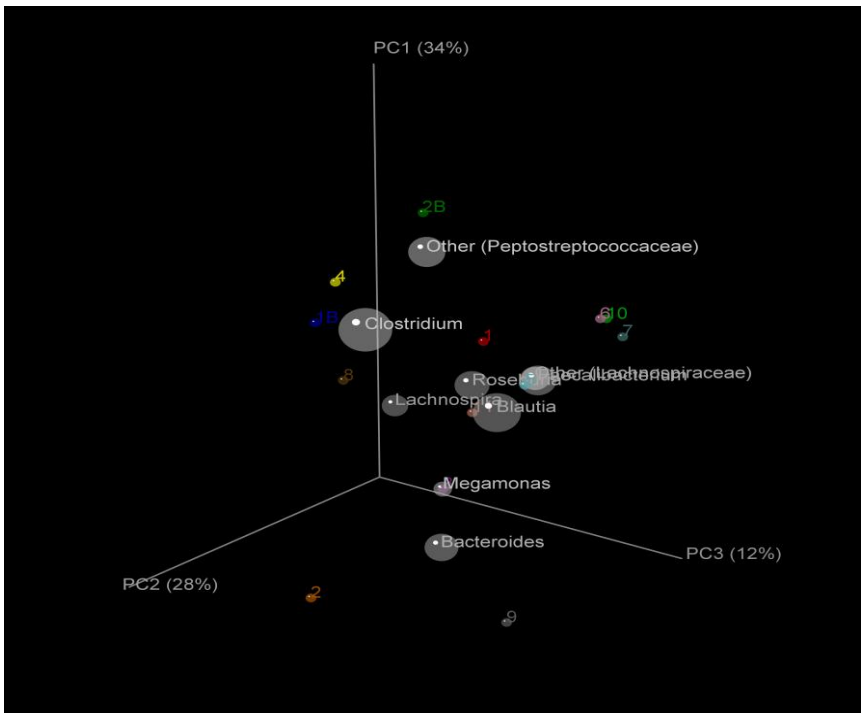


Figure 4. 3D Bi-Plot of the principal coordinate analysis on weighted UniFrac distance matrix. The ten most abundant taxa at genus level were plotted with circles proportional to their abundance. Patients were numbered 1-11. Patients 12-14 were excluded due to the low number of sequences (<500). B denotes sample taken one month after surgery.