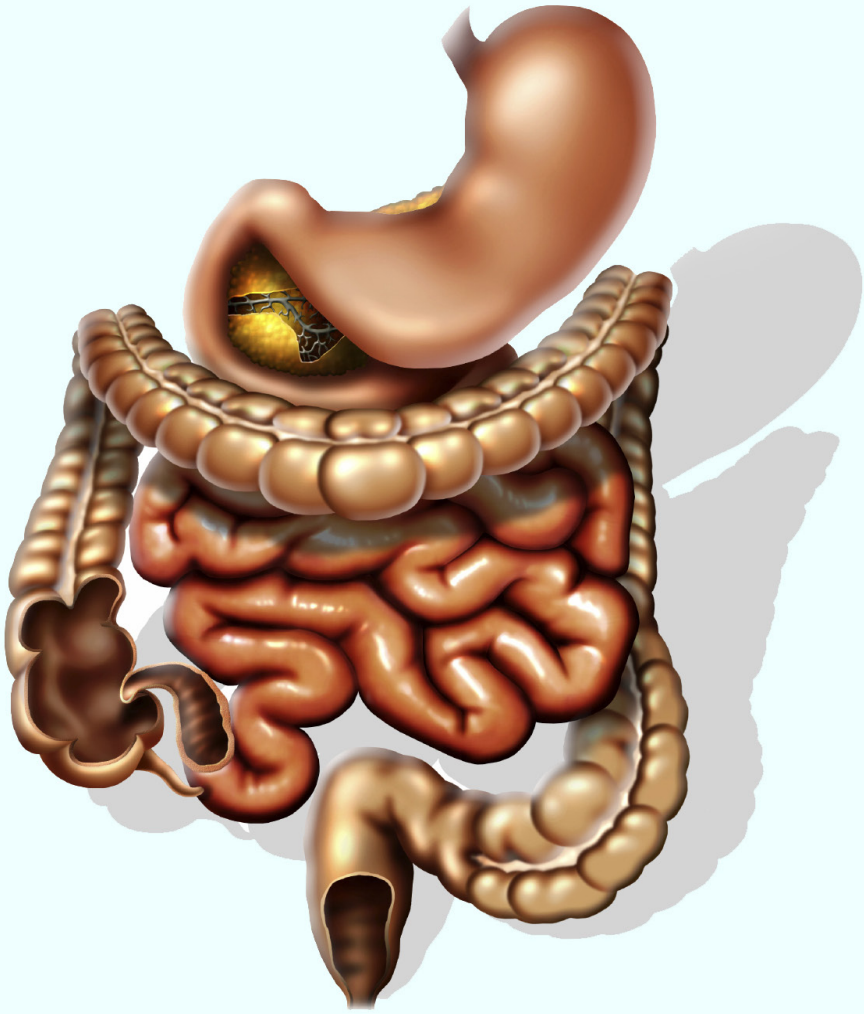

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Dissertation
45

Dietary effects on human fecal microbiota

Catarina Simões

VTT

VTT SCIENCE 45

Dietary effects on human fecal microbiota

Catarina Simões

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Dietary effects on human fecal microbiota

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Abstract

The establishment of microbial populations in the gastrointestinal (GI)-tract is a complex process, involving microbial and host interactions eventually resulting in a dense and stable population. Recently, the identification of microbial species from fecal samples has become more accurate with the use of 16S RNA gene-based methods. However, although these molecular-based detection methods have apparent benefits over culture-based techniques, they involve potential pitfalls that should be taken into consideration when studying the fecal microbiota, such as the storage conditions and deoxyribonucleic acid (DNA)-extraction. Therefore, the effects of different storage conditions and DNA-extraction protocols on fecal samples were evaluated in this study. Whereas the DNA-extraction protocol did not affect the numbers of *Bacteroides* spp., the abundance of this group showed a significant decrease after one week's storage at -20°C. Furthermore, the numbers of predominant bacteria, *Eubacterium rectale* group, *Clostridium leptum* group, bifidobacteria and *Atopobium* group, were significantly higher in samples stored at -70°C after mechanical DNA-extraction than after enzymatic DNA-extraction as detected with real-time PCR (qPCR). These results indicate that rigorous mechanical lysis leads to the detection of higher bacterial numbers from human fecal samples than enzymatic DNA-extraction. Therefore, the use of different DNA-extraction protocols may partly explain contradictory results reported in previous studies.

The composition of the human intestinal microbiota is influenced by host-specific factors such as age, genetics and physical and chemical conditions encountered in the GI-tract. On the other hand, it is modulated by environmental factors with impact on the host during the lifespan, such as diet. The impact of diet on the gut microbiota has usually been assessed by subjecting people to the same controlled diet, and thereafter following the shifts in the microbiota. In the present study, the habitual dietary intake of monozygotic twins was associated with the fecal microbiota composition, which was analysed using qPCR and Denaturing Gradient Gel Electrophoresis (DGGE). The effect of diet on the numbers of the bacteria was described using a hierarchical linear mixed model that included the twin individuals, stratified by body mass index, and their families as random effects. The abundance and diversity of the bacterial groups studied did not differ between normal weight, overweight, and obese individuals with the techniques used. However, intakes of energy, monounsaturated fat, (n-3) polyunsaturated fat, (n-6) polyunsaturated fat and soluble fibre had significant associations with the fecal bacterial numbers. In addition, co-twins with identical energy intakes had more similar numbers and DGGE-profile diversities of *Bacteroides* spp. than co-twins

with different intakes. Moreover, co-twins who ingested the same amounts of saturated fat had very similar DGGE-profiles of *Bacteroides* spp., whereas co-twins with similar consumption of fibre had very low bifidobacterial DGGE-profile similarity.

Thereafter, the impact of the energy intake on the fecal microbiota of a group of 16 obese individuals was assessed during a 12 month intervention, which consisted of a 6 week very low energy diet (VLED) and thereafter a follow-up period of 5, 8 and 12 months. The diet plan was combined with exercise and lifestyle counseling. Fecal samples were analyzed using qPCR, DGGE and fluorescent *in situ* hybridization. The effect of the energy restricted diet on the fecal bacterial numbers was described using a linear mixed model that accounted for repeated measurements in the same individual. The VLED period affected the major fecal microbial groups; in particular bifidobacteria decreased compared to the baseline numbers. Methanogens were detected in 56% of the participants at every sampling time point, regardless of the change in dietary intake. Furthermore, the change in numbers of the fecal bacterial groups studied followed the dietary intake and not the weight changes during the 12 months. These findings confirm that the diet and energetic intake play an important role in modulation of the fecal microbiota.

Finally, the potential of utilising the information on expression levels of selected stress genes in assessing the quality of probiotic products was evaluated. For this purpose, reverse transcription-qPCR methods were developed to study the expression of c*lpL1* and c*lpL2* stress genes in *Lactobacillus rhamnosus* VTT E-97800 cells after exposure to processing-related stress conditions or to freeze-drying. Heat treatments were performed with *L. rhamnosus* VTT E-97800 in laboratory scale, whereas acid treatments were performed both in laboratory and fermenter scale. RNA was extracted from fresh cells and freeze-dried powders. *clpL1* and *clpL2* transcripts were analysed by qPCR using SYBR Green I. *clpL1* was induced in *L. rhamnosus* VTT E-97800 cells exposed to 50°C and to a much lesser extent in cells exposed to 47°C. No induction was observed for *clpL2* during either acid or heat treatment in any of the conditions applied. RNA isolation from freeze-dried powders was unsuccessful, although several attempts were made with high quality products. These results suggest that developing quality indicators for probiotic products based on differences in the expression of stress genes will be a challenging task, since rather harsh conditions are apparently needed to detect differences in the gene expression. In addition, the unsuccessful RNA isolation from freeze-dried powders hampers the applicability of this technique in the quality control of probiotic products.

Keywords human fecal microbiota, DNA-extraction, diet, very low energy diet, qPCR, stress response

Preface

The research work presented in this dissertation was carried out at VTT Technical Research Centre of Finland in Espoo during the years 2008 to 2012, and at the Rowett Institute for Nutrition and Health, University of Aberdeen, Scotland, during the period of 18th April to 20th May 2011.

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Catarina Simões

Academic dissertation

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List of publications

This thesis is based on the following original publications, which are referred to in the text as I–IV. In addition, some unpublished data is also presented. The publications are reproduced with permission from the publishers.

I Maukonen, J., Simões, C. & Saarela, M. 2012. The current used commercial DNA extraction methods give different results of clostridial and actinobacterial populations derived from human fecal samples. *FEMS Microbiology Ecology* 79(3):697–708.

II Simões, C.D., Maukonen, J., Kaprio, J., Rissanen, A., Pietiläinen, K.H. & Saarela, M. 2013. Habitual dietary intake is associated with the stool microbiota composition of monozygotc twins. *Journal of Nutrition* 143(4):417–423.

III Simões, C.D., Maukonen, J., Scott, K.P., Virtanen, K.A., Pietiläinen, K.H. & Saarela, M. Impact of a very low energy diet in the fecal microbiota of obese individuals. Manuscript submitted.

IV Simões, C., Alakomi, H.-L., Maukonen, J. & Saarela, M. 2010. Expression of *clpL1* and *clpL2* in *Lactobacillus rhamnosus* VTT E-97800 after exposure to acid and heat stress treatments or freeze-drying. *Beneficial Microbes* 1(3): 253–257.

Author's contributions

I The author performed part of the experimental work, including optimization of qPCR methods, analysis of qPCR data and interpretation of the results.

II The author performed part of the experimental work, developed statistical models to analyse the data and interpreted the results. The author wrote the article and is the corresponding author.

III The author planned and performed the experimental work. Part of the experimental work was performed at the Rowett Institute for Nutrition and Health, University of Aberdeen, UK under the supervision of Dr. Karen Scott. The author analysed the data, interpreted the results, wrote the manuscript and is the corresponding author.

IV The author planned the study together with Dr. Hanna-Leena Alakomi, performed the experimental work, analysed the data, wrote the article and is the corresponding author.

Contents

List of abbreviations

ATCC	American Type Culture Collection
BMI	body mass index
bp	base pair
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
CH_4	methane
CO_2	carbon dioxide
ct	crossing point
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)
E800	VTT E-97800
FISH	fluorescent *in situ* hybridization
G+C	guanine plus cytosine
GEM	general edible medium
GI	gastrointestinal
H_2	hydrogen
LAB	lactic acid bacteria
MRSA	de Man, Rogosa and Sharpe Agar
MUFA	monounsaturated fat
MZ	monozygotic
NCBI	National Centre for Biotechnology Information

PUFA	polyunsaturated fat
qPCR	quantitative real-time polymerase chain reaction
RDP	Ribosomal Database Project
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	reverse transcription
SCFA	short-chain fatty acids
SFA	saturated fat
VLED	very low energy diet
WHO	World Health Organization

1. Introduction

Humans host several communities of microorganisms located in different parts of the body such as mouth, skin, vagina and the gastrointestinal (GI)-tract. The GI microbiota is the microbial community inhabiting the GI-tract (Gordon, 2012), containing approximately ten times more prokaryotic cells than the total number of eukaryotic cells within the human body (Savage, 1977). The members of the microbiota represent an aggregate biomass of around 1.5 kg (Xu and Gordon, 2003) and a metagenome encoding at least 100 times as many genes as the complete human genome (Turnbaugh et al., 2009b).

The GI-tract microbes are often described as commensals. Commensalism refers to a relationship with benefits to one partner while the other is unaffected (Hooper and Gordon, 2001). However, the interaction between the microbiota and the human host has been defined as a symbiotic relationship (where both partners benefit), mutually beneficial in a healthy environment (Hooper et al., 1998, Ley et al., 2006a). The host provides a stable and nutrient-rich habitat, while the microbiota confer important benefits to the host. The intestinal microbiota ferments non-digested dietary components and endogenous mucus produced by the intestinal mucosa, with production of short chain fatty acids (SCFA), amino acids and vitamins. In addition, the microbiota resists colonization of pathogens by preventing invasion by exogenous organisms e.g. through the production of metabolites and bacteriocins (Wells, 2008), and affects the development and homeostasis of the intestinal epithelium and the immune system (Guarner, 2008).

The composition of the human intestinal microbiota is influenced by host factors such as age, genetics, secretory products such as gastric acid and bile, peristalsis and GI transit time (Spor et al., 2011, Parfrey and Knight, 2012). On the other hand, environmental factors with impact on the host during the lifespan, such as diet, continuously modulate this microbial community.

1.1 Digestive system

Unlike plants, which can form organic molecules using inorganic compounds, humans and other animals obtain their essential biomolecules from the diet. The digestive system affects the processing and digestion of the food ingested. The overall digestive system is anatomically and functionally divided into the GI tract,

or alimentary canal, and its accessory digestive organs (Figure 1). The tubular GI-tract is approximately nine meters long and extends from the mouth to the anus. It transverses the thoracic cavity and enters the abdominal cavity at the level of the diaphragm. Its organs include the oral cavity, pharynx, esophagus, stomach, small intestine and large intestine. The anus is the external opening of the anal canal, located at the bottom of the pelvic cavity, through which the waste materials, or feces, are excreted. The lumen of the GI-tract is open at both ends, so it is continuous with the environment. This feature permits one-way transport of the ingested food, ensured by wavelike muscle contractions (peristalsis) and by the action of sphincter muscles, which allows different regions of the GI-tract to be specialized for different functions (Fox, 1999). The accessory digestive organs include the teeth, tongue, salivary glands, liver, gall bladder and pancreas.

Although the physiological role of the GI-tract is to process and digest the food ingested, it also offers several niches for colonization by a variety of microorganisms (Marchesi, 2011). The numbers of microbes increase distally from the stomach to the large intestine, which contains one of the densest communities known with about 10^{12} bacteria per gram (Gillilland et al., 2012).

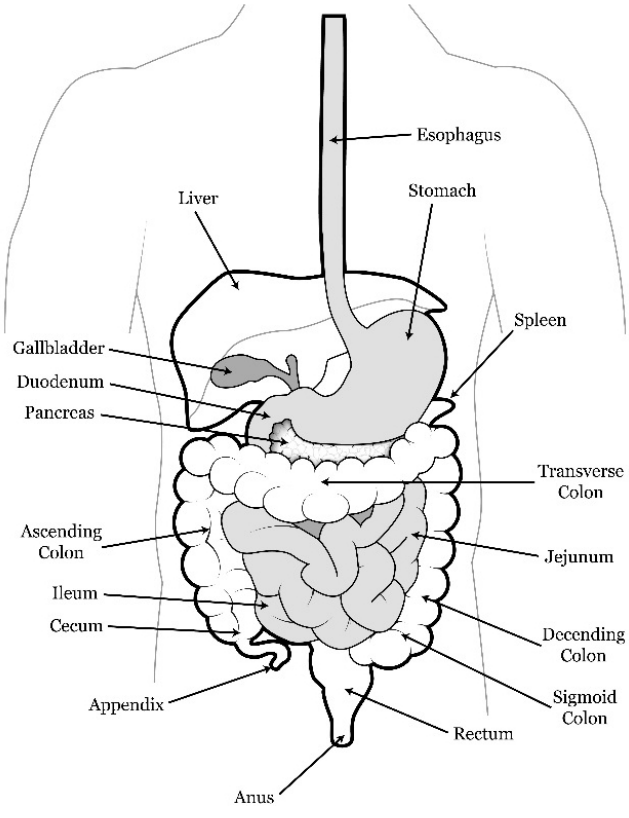

Figure 1. Representation of the human gastrointestinal tract.

1.1.1 Upper gastrointestinal tract

The mechanical and enzymatic digestion of food starts in the oral cavity. Here, tongue and teeth break down the food materials into smaller particles and masticate them with saliva. Saliva is secreted by the salivary glands and contains mucus, bicarbonate and antimicrobial agents and is rich in enzymes. Amylase, produced in small amounts in saliva, catalyses the partial digestion of starch (Pedersen et al., 2002). Although there is no clear evidence of the importance of oral microbiota in the digestive process, the microorganisms inhabiting the mouth have a role in inoculating the rest of the GI-tract (Yilmaz, 2008).

Deglutition begins as voluntary activity, during which the larynx raises so that the epiglottis covers the entrance to the respiratory tract. Swallowed food is then pushed along the esophagus into the stomach. Six different bacterial phyla have been identified in distal esophageal biopsies (Pei et al., 2004). Firmicutes was the most prevalent phylum represented, followed by Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria and TM7.

The stomach is continuous with the esophagus and empties into the duodenum of the small intestine. The stomach has a low pH, typically pH 2 during fasting conditions. The low pH of the gastric juice represents a barrier to many microorganisms on their route to establish themselves in the intestine. However, most bacteria have adaptive mechanisms that can confer high levels of acid tolerance (Foster, 2004). In addition, the stomach pH varies considerably, reaching values up to pH 5 after a large meal, which might contribute to the occasional passage of live bacterial cells from the stomach to the duodenum. On the other hand, the low pH of the gastric juice produces an indirect antimicrobial effect through the production of nitrous acid from nitrite, which is ingested along with food or produced in the mouth from nitrate fermentation by the oral microbiota (Louis and O'Byrne, 2010). The nitrous acid spontaneously decomposes in the stomach, producing reactive nitrogen species with potent antimicrobial activity on for example enteropathogens (Benjamin et al., 1994). Overall, the stomach plays an important role in shaping the microbiota downstream in the lower GI-tract.

Although the bacterial load of the stomach's content is very low in healthy adults, approximately $10^{2\text{-}3}$ colony forming units (CFU) per ml content (O'May et al., 2005), its mucosal surface is colonized with microorganisms. Molecular analyses of the human stomach mucosa have surprisingly revealed high gastric bacterial diversity. Members of the phyla Proteobacteria (mainly the ε-proteobacteria *Helicobacter pylori*), Firmicutes and Bacteroidetes are predominant in gastric biopsies, while members of the phyla Actinobacteria, Fusobacteria, Deferribacteres and *Deinococcus/Thermus* occur in lower proportions (Bik et al., 2006). Moreover, viable lactic acid bacteria (LAB), in particular *Lactobacillus reuteri, Lactobacillus salivarius* and *Streptococcus salivarius*, have been recovered from gastric biopsies of healthy patients (Hakalehto et al., 2011).

Besides being a barrier to microorganisms, other functions of the stomach include food storage, initiation of the digestion of proteins and transfer of food into the small intestine as a pasty material called chyme.

1.1.2 Lower gastrointestinal tract

The small intestine comprises the duodenum, jejunum and ileum (Figure 1). The arrival of chyme from the stomach into the duodenum causes contraction of the gall bladder and ejection of bile. Bile is produced in the liver, stored in the gall bladder, and secreted into the duodenum upon ingestion of a meal. Bile emulsifies lipo-soluble dietary components, promoting their digestion and absorption. The major constituents of bile are the bile salts, bilirubin, phospholipids, cholesterol, and inorganic ions. Deconjugated bile salts are normally present in the intestinal content and may affect the composition of the colonic microbiota (Savage, 1977). Due to their amphipathic properties, bile acids interact with the membrane phospholipids, damaging the bacterial cells (Yokota et al., 2012). In addition to bile, food is blended in the duodenum with bicarbonate and digestive enzymes.

Due to the aggressive intestinal fluids and the short transit time, the duodenum represents a hostile environment containing relatively low numbers of transient microbes (Holzapfel et al., 1998). Common bacterial inhabitants of the duodenum are *Streptococcus* spp., *Lactobacillus* spp., bifidobacteria, *Staphylococcus* spp. and the Enterobacteriaceae (O'May et al., 2005).

Most of the digestion and absorption of the food nutrients occurs in the small intestine. The lumen of the small intestine is lined with simple columnar epithelial cells covered by a mucus layer formed by villi (Rubin, 2009). Villi increase the surface area of the small intestine and secrete enzymes that aid in the digestion and absorption of carbohydrates, proteins and lipids. Chyme moves along the small intestine by rhythmic and wave-like muscular contractions called peristalsis (Schiller, 1999). The rate of peristalsis greatly influences the microbiota composition. Peristalsis moves unattached bacteria along the intestine, preventing colonisation of the epithelial surface (Savage, 1978) and bacterial overgrowth (Li, 1995). At the same time, both endocrine and exocrine secretions such as water, hydrochloric acid, bicarbonate and many digestive enzymes are released into the lumen of the intestine to improve the breakdown of the dietary material (Booijink et al., 2007). A major part of the digestible food components is absorbed when the digesta reaches the terminal ileum (Saulnier et al., 2009). The remaining undigested food materials not degraded and absorbed in the small intestine now reach the large intestine, where they support the microbiota as a source of nutrients and energy (Leser and Molbak, 2009).

In the jejunum and ileum, the microbiota composition is more diverse than in the duodenum and is composed of both anaerobes and facultative anaerobes. As the transit time slows in the ileum, the pH increases and the oxidation-reduction potential decreases, and the microbiota switches to increasing numbers of anaerobic species (Dethlefsen et al., 2006), with total bacterial counts of approximately

10^6-10^8 CFU per ml content (Kerckhoffs et al., 2006). Studies in human biopsies of the jejunum and distal ileum have reported that whereas in jejunal mucosa the *Bacillus/Lactobacillus/Streptococcus* group and Proteobacteria were the most abundant groups, in distal ileal mucosa the Bacteroidetes and *Clostridium* were dominant (Wang et al., 2005). Furthermore, in ileal effluent samples from subjects with ileostomy, the class Clostridia was dominant in all individuals, whereas streptococci, ruminococci and eubacteria were detected in most of the individuals (Booijink et al., 2010). However, due to difficulties in sampling, the microbiota composition of the human small intestine is poorly known.

The large intestine extends from the end of the ileum to the anus. It receives a daily volume of 0.5–2.5 L of chyme from the terminal ileum into the cecum that sequencially passes through the ascending colon, transverse colon, descending colon, sigmoid colon, rectum and anal canal (Cohn and Birnbaum, 1995). The large intestine differs from the small intestine in its greater calibre, more fixed position, sacculated form, and in possessing certain appendages to its external coat. The mucosa of the large intestine, like that of the small intestine, contains scattered lymphocytes, lymphatic nodules and is covered by columnar epithelial cells and mucus-secreting goblet cells. Although this epithelium does form crypts of Lieberkühn, there are no villi in the large intestine and therefore its mucosa appears flat. The mucus layer is composed of a water-insoluble gel that forms a protective stable layer over the surface of the delicate GI epithelium, protecting it from harmful substances within the lumen such as gastric acid, digestive enzymes such as pepsin and trypsin, ingested toxins or co-carcinogens, bacterial toxins and enzymes, and oxygen-derived free radicals (Cross et al., 1984, Allen et al., 1988). The glycoprotein mucin provides the gel-forming properties of the mucus barrier and is the major host-derived source of carbohydrates in the intestine. A limited mucolytic bacterial community exists in the human GI-tract, including a recently described bacterium *Akkermancia muciniphila*, a member of the phylum Verrucomicrobia (Derrien et al., 2008).

The transit of chyme through the large intestine is slow, varying from 10 h to several days, compared to a few hours of transit time through the small intestine. The slow transit time and the vigorous mixing movements aid the two major functions of the large intestine: microbial fermentation and the reabsorption of water and electrolytes (Smith and Sanders, 1995). Low transit rates encourage the growth of slower growing microorganisms, including some of the hydrogen-utilizers such as methanogens (El Oufir et al., 2000). *Methanobrevibacter smithii* is the most abundant methanogenic archaea in the human colon, while *Methanosphaera stadtmanae* is present in lower proportions of individuals (Dridi et al., 2009). In addition, there is evidence for an age-related increase in the proportions of subjects presenting *MX*-phylotypes in their GI-tract that may be explained by the increased transit time due to a reduction of fecal weight in elderly (Mihajlovski et al., 2010). Methanogens increase the efficiency of bacterial fermentation in the large intestine by utilizing one of the end products. Methane (CH_4) production consumes 4 mol of H_2 to reduce 1 mol of CO_2 (Sahakian et al., 2010). This process greatly reduces the volume of colonic gas. Since CH4 is not utilized by humans, it is excreted either as

flatus, or traverses the intestinal mucosa and is absorbed into the systemic circulation and then excreted through the lungs. The measurement of breath CH_4 is often used as an indirect measure of its production rate in the large intestine (Eckburg et al., 2003). The concentration of CH_4 is extremely variable, and like hydrogen sulphide, is not present in all individuals. Other human intestinal gases include nitrogen, oxygen, carbon dioxide (CO_2) and hydrogen (H_2).

The pH of the intestinal lumen is determined by host secretions and the fermentation products of the gut microbiota (Duncan et al., 2009). The fermentation of dietary substrates mainly occurs in the proximal colon, resulting in active production of SCFA with consequent reduction of the pH in this segment of the colon (Macfarlane et al., 1992). In the distal colon and feces, the pH is higher and closer to neutral. Several studies have reported considerable variations in responses to pH among the phylogenetic groups of human colonic bacteria (Duncan et al., 2009, Walker et al., 2005). For example *Bacteroides* spp., the major Gram-negative bacterial group within the human gut, is sensitive to acidic pH and its levels increase with increasing pH from 5.5 to 6.5. Therefore, variations in pH affect the microbial community composition and metabolic activity (Duncan et al., 2009).

1.2 Microbiota in the human large intestine

1.2.1 Establishment of the microbiota

At the time of birth the human intestine is thought to be aerobic (Fouhy et al., 2012) and sterile (Palmer et al., 2007). Within the first few days of life, the infant GI-tract becomes densely colonized, mainly with bacteria environmentally exposed to the baby (Palmer et al., 2007). Facultative anaerobes such as *Staphylococcus*, *Streptococcus* and Enterobacteria colonize the infant´s colon soon after birth, followed by strict anaerobic genera such as *Eubacterium, Clostridium*, *Bifidobacterium* and *Bacteroides* (Palmer et al., 2007, Mariat et al., 2009, Salminen and Isolauri, 2006). However, the delivery mode influences the infant's intestinal microbiota composition (Fallani et al., 2010). Early culture-based microbiological studies suggested that babies acquire their initial microbiota from the vagina and feces of their mothers (Mandar and Mikelsaar, 1996). In fact, babies delivered by caesarean section show an altered colonization pattern as compared to their vaginally-delivered counterparts (Gronlund et al., 1999). Vaginal delivery is associated with earlier colonization of *Bacteroides* spp. (Adlerberth et al., 2006) and higher proportions of *Atopobium* spp., as compared to caesarean section that is associated with higher *E. rectale-B. coccoides* and *Streptococcus* groups (Fallani et al., 2010, Fallani et al., 2011, Gronlund et al., 1999). In addition, vaginally delivered infants are associated with higher prevalence of *E. coli* when compared to caesarean section (Adlerberth et al., 2006). Early studies claim that *Bacteroides* spp. appear in the newborn approximately 10 days after birth (Simon and Gorbach, 1984). On the other hand, other studies show that, although consistently present in the feces of most babies, *Bacteroides* spp. vary between individuals in

the timing of their first appearance (Palmer et al., 2007). A recent metagenomics study showed that infants acquire undifferentiated bacterial communities across the body, however, while the fecal microbiota of vaginally delivered infants resemble their own mother's vaginal microbiota dominated by *Lactobacillus*, *Prevotella* or *Sneathia* spp., caesarean section infants harbor bacterial communities similar to those found on the skin surface, dominated by *Staphylococcus*, *Corynebacterium* and *Propionibacterium* spp. (Dominguez-Bello et al., 2010).

Regarding bifidobacteria, numerous studies have concluded that this group of bacteria dominates the infant fecal microbiota (Harmsen et al., 2000b; Favier et al., 2002; Magne et al., 2006; Fallani et al., 2010; Mueller et al., 2006). By contrast, another study found bifidobacteria to constitute only a minor part of the infant microbiota (Palmer et al., 2007), although the deoxyribonucleic acid (DNA) extraction method applied in this study was not optimal for detection of Gram-positive bacteria (Maukonen et al., 2012). *Bifidobacterium longum* and *Bifidobacterium bifidum* were the dominant bifidobacterial species found in intant feces (Turroni et al., 2012). Although the genus *Bifidobacterium* dominates the fecal microbiota throughout the first year of life, its proportional representation decreases during this period (Yatsunenko et al., 2012, Favier et al., 2002), due to the increase in the numbers of other anaerobic bacteria.

The feeding pattern also influences the composition of the microbiota of the infant's large intestine. Breast-fed infants have a higher abundance of bifidobacteria in their feces as compared to formula-fed infants (Mackie et al., 1999, Fallani et al., 2010). Breast-feeding provides oligosaccharides that promote the growth of bifidobacteria in the infant gut (Salminen and Isolauri, 2006). By contrast, cow's milk-based formulas, which lack the amount and diversity of oligosaccharides present in human milk, facilitate the colonization of different intestinal microorganisms during infancy (Koropatkin et al., 2012). Formula-fed infants have greater proportions of *Bacteroides* spp., *E. rectale-B. coccoides* and *Lactobacillus* groups in their feces compared to breast-fed infants (Fallani et al., 2010). This is a clear indication that diet can influence the abundance of species within the intestinal microbiota (Holzapfel et al., 1998).

After introduction of the first solid foods, the predominant fecal bacterial groups were bifidobacteria, followed by *B. coccoides* and *Bacteroides* (Fallani et al., 2011). During post-weaning, the carbohydrate composition of the diet undergoes an abrupt change when more complex foods such as cereals, fruits and vegetables are introduced in the infant's diet. When such polymers reach the intestine the composition of the microbiota shifts, and microorganisms that are able to degrade complex carbohydrates become more prevalent (Koenig et al., 2011).

1.2.2 Spatial distribution of the microbiota

Localization of the microbiota in the adult human large intestine has been discussed for many years (Dubos et al., 1965). It is generally accepted that the microbiota is divided into two groups according to their location: the autochthonous

or resident microbes and the allochthonous or transient microbes. The resident microbes inhabit physical niches in the ecosystem that are thought to be closely related to the intestinal mucosa (Savage, 1977, Nava and Stappenbeck, 2011). They are tolerated by the immune system of the host and form stable populations for long-term periods (Walter and Ley, 2011). Therefore, the resident community must be highly compatible with the intestinal environment (Tannock, 1999). On the other hand, the transient microbes do not establish, colonize or multiply in the large intestine. As they are just passing through the GI-tract, they are thought to be located only in the lumen as part of the fecal stream (Tannock, 1999). These microorganisms may derive from other habitats of the human body such as skin, mouth and upper respiratory tract, or from environmental sources such as water and food (Ley et al., 2006a). Despite the continuous flow of colonic contents and the opportunity for microbes to be constantly washed out, the microbial density and diversity in the colon is high and remains relatively stable over time (Leser and Molbak, 2009). This homeostasis suggests that the microbial community present in the colon is to a large extent dominated by the resident microbiota (Walter and Ley, 2011). In a perturbed GI-tract or under abnormal conditions transient microbes may colonize vacated niches, leading to the development of diseases (Savage, 1977). Specific imbalances or deviations in the intestinal microbiota may make humans more susceptible to intestinal inflammatory and systemic diseases. Some pathogens are autochthonous to the GI ecosystem and live in harmony with their hosts, becoming pathogenic only when the ecosystem is disturbed (Mackie, 1997) and their numbers increase.

Due to the physiology of the colon, the resident microbes commonly found in the adult large intestine share the same general features. They should be capable of growing anaerobically, with an optimal growth temperature around 37°C, and have the capacity to grow quickly enough to avoid washout (Savage, 1977). The resident microbes colonize particular areas of the intestine, maintaining stable population levels in normal adults (Mackie, 1997). Although they may interact with the mucosa in the areas colonized, they are not immunogenic in the host's GI-tract.

1.2.3 Composition of the microbiota in the adult large intestine

The composition of the intestinal microbiota diversifies after the first few years of life, eventually converging into an adult-like phylogenetic structure (Palmer et al., 2007, Yatsunenko et al., 2012). The microbiota of the adult large intestine is constituted by a small number of phyla which belong to the three domains of the Tree of Life: Bacteria, Archaea, and Eukarya. However, 99% of the sequenced genes from fecal samples belong to Bacteria (Qin et al., 2010). The phylogeny of microbial genera present in human feces according to the National Centre for Biotechnology Information (NCBI) taxonomy is presented in Table 1 (Sayers et al., 2009).

Within recent decades, the small subunit of the ribosomal ribonucleic acid (rRNA) gene has been widely used in culture-independent studies of gut microbiota. The 16S and 23S rRNA genes are considered to be excellent phylogenetic markers

since they have universal distribution, structural and functional conservation, although containing both fast- and slow-evolving regions (Ludwig and Schleifer, 1994, Zaneveld et al., 2010). In contrast to the traditional taxonomy based on phenotypic traits, rRNA taxonomy reflects the genomic evolution among prokaryotes and archeae (Woese et al., 1990). 16S rRNA-based analyses have allowed the construction of species phylogenetic trees based on 16S rRNA databases such as the Ribosomal Database Project (RDP) (Cole et al., 2009) and SILVA rRNA database project (Quast et al., 2013). Therefore, 16S rRNA-targeted primers and probes have been designed to target specific phyla, specific groups, or major species, within the intestinal microbiota (Franks et al., 1998, Matsuki et al., 2002, Lay et al., 2005b, Muyzer et al., 1993, Matsuki et al., 2004a). By analysing mixtures of rRNA genes it is possible to phylogenetically identify population members such as in the gut microbial community (Olsen et al., 1986).

According to 16S rDNA-based studies, the dominant bacterial phyla within the human fecal microbiota are Firmicutes (39–76%), Bacteroidetes (17–28%) and Actinobacteria (2.5–8%) (Tap et al., 2009, Eckburg et al., 2005, Arumugam et al., 2011, Andersson et al., 2008). In addition, lower amounts of Proteobacteria (2.1%), Verrucomicrobia (1.3%), Euryarchaeota (0.9%), and Fusobacteria have been identified in human fecal samples (Arumugam et al., 2011). Studies using 16S rRNA gene-based phylogenetic microarrays confirm that Firmicutes are the most abundant phylum within the fecal microbiota of healthy individuals, followed by Bacteroidetes and Actinobacteria (Jalanka-Tuovinen et al., 2011, Rajilic-Stojanovic et al., 2009).

Within the plylum Firmicutes, the class Clostridia has been recognized as dominant in several studies (Eckburg et al., 2005, Andersson et al., 2008, Gill et al., 2006, Jalanka-Tuovinen et al., 2011, Rajilic-Stojanovic et al., 2009). Phylogenetic microarray studies identified approximately 75% of the microbiota as members of the families Lachnospiraceae (40%) and Ruminococcaceae (35%). According to 16S rRNA gene-targeted FISH studies (Lay et al., 2005a, Rigottier-Gois et al., 2003, Lay et al., 2005b), 22–28% of the total bacterial cells belong the *Eubacterium rectale – Blautia coccoides* group (or *E. rectale* group; family Lachnospiraceae), while 22–26% of the bacterial cells are members of the *Clostridium leptum* group (part of the Family Ruminococcaceae). *Bacteroides* spp. represent around 9% of the total fecal bacteria, followed by members of the *Atopobium* group and bifidobacteria with approximately 3.5% and 4% of total bacterial cells, respectively. The interindividual variation between samples is high.

Table 1. Phylogeny of the microbial genera present in human feces, according to the National Centre for Biotechnology Information taxonomy.

Phylum	Class	Order	Family	Genus
Firmicutes	Clostridia	Clostridiales	Clostridiaceaea	*Clostridium*
				Sarcina
			Eubacteriaceae	*Eubacterium*
			Lachnospiraceae	*Anaerostipes*
				Blautia
				Butyrivibrio
				Coprococcus
				Dorea
				Lachnospira
				Roseburia
				Ruminococcus
			Peptococcaceae	*Peptococcus*
			Peptostreptococcacea	*Peptostreptococcus*
				Clostridium
			Ruminococcacea	*Anaerofilum*
				Anaerotruncus
				Clostridium
				Faecalibacterium
				Ruminococcus
				Subdoligranulum
	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*
			Bacillaceae	*Bacillus*
		Lactobacillales	Enterococcaceae	*Coprobacillus*
				Enterococcus
			Lactobacillaceae	*Lactobacillus*
				Pediococcus
			Leuconostocaceae	*Leuconostoc*

					Weissella
				Streptococcaceae	*Streptococcus*
					Lactococcus
			Selenomonadales	Veillonellaceae	*Megasphaera*
					Pectinatus
					Veillonella
				Acidaminococcaceae	*Acidaminococcus*
		Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	*Erysipelotrix*
	Bacteroidetes	Bacteroidia	Bacterioidales	Bacteroidaceae	*Bacteroides*
				Porphyromonadaceae	*Parabacteroides*
				Rikenellacea	*Alistipes*
				Prevotellacea	*Prevotella*
	Actinobacteria	Actinobacteridae	Bifidobacteriales	Bifidobacteriaceae	*Bifidobacterium*
					Scardovia
		Coriobacteridae	Coriobacteriales	Coriobacteriaceae	*Atopobium*
					Collinsella
					Coriobacterium
					Eggerthella
					Slackia
	Proteobacteria	δ-proteobacteria	Desulfovibrionales	Desulfovibrionaceae	*Desulfovibrio*
		γ-proteobacteria	Enterobacteriales	Enterobacteriaceae	*Echerichia*
					Enterobacter
	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	*Akkermansia*
	Fusobacteria	Fusobacteria	Fusobacteriales	Fusobacteriaceae	*Fusobacterium*
	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	*Methanobrevibacter*
					Methanosphaera

The *E. rectale* group represents the family Lachnospiraceae, comprising genera closely related to *E. rectale* (genus *Lachnospiraceae*_incertae_sedis according to RDP) such as *Anaerostipes*, *Blautia*, *Dorea* and *Roseburia* (Wang et al., 2007). The main products of fermentation of sugars produced by bacteria within this group include acetate, butyrate, lactate, formate and succinate. Butyrate-producers from carbohydrate fermentation include species such as *Roseburia* spp., *E. rectale* and *Eubacterium hallii* (Louis and Flint, 2007). Furthermore, a number of bacterial

species including *Anaerostipes caccae* and *E. hallii* utilise lactate through cross-feeding, producing acetate and butyrate (Duncan et al., 2004b).

The *C. leptum* group belongs to the family Ruminococcaceae, comprising phylogenetically related species to *C. leptum* (genus *Clostridium* IV according to RDP). The bacteria within the *C. leptum* group are saccharolytic and the main end-products of fermentation are lactate, acetate and butyrate. Butyrate-producers belong to the genera *Faecalibacterium* (Duncan et al., 2002), *Subdoligranulum* (Holmstrom et al., 2004) and *Anaerotruncus* (Lawson et al., 2004). *Faecalibacterium prausnitzii* is one of the most abundant species detected within the *C. leptum* group (Suau et al., 1999, Suau et al., 2001, Arumugam et al., 2011, Rigottier-Gois et al., 2003). The relative abundance of *F. prausnitzii* is reduced in certain forms of inflammatory bowel disease (Sokol et al., 2009, Cucchiara et al., 2009), colorectal cancer (Balamurugan et al., 2008) and in frail elderly people (Mariat et al., 2009, Van Tongeren et al., 2005), suggesting that this bacterium could provide an indicator of a healthy intestinal microbiota.

The *Lactobacillus* group comprises genera closely related to *Lactobacillus* spp. within the phylum Firmicutes. *Lactobacillus* spp. comprise Gram-positive rods or cocobacilli with low cytosine plus guanine (C+G) content, and are non-spore-forming, facultatively anaerobic and strictly fermentative (Claesson et al., 2007). The main end product of carbohydrate metabolism is lactic acid, in addition to acetate, ethanol, CO_2, formate, or succinate, depending on the type of fermentation. *Lactobacillus* spp. have a long application history in the food industry, contributing to the production of e.g. cheese, yogurt and other fermented products.

Within the phylum Bacteroidetes, *Bacteroides* is the most abundant genus inhabiting the human intestine (Andersson et al., 2008, Karlsson et al., 2011). *Bacteroides* spp. comprise rod-shaped Gram-negative bacteria with a low G+C content of 40–48 mol%, and are obligate anaerobes and non-spore-forming. Members of the *Bacteroides* spp. are saccharolytic and their main end-products of fermentation are acetate, propionate and succinate (Chaudhry and Sharma, 2011). *Bacteroides vulgatus, Bacteroides distasonis, Bacteroides thetaiotaomicron, Bacteroides fragilis, Bacteroides ovatus, Bacteroides coprocola* and *Bacteroides uniformis* have been found in human fecal samples (Li et al., 2009, Salyers, 1984).

Within the phylum Actinobacteria, the *Atopobium* group (i.e. Family Coriobacteriaceae) comprises genera such as *Atopobium*, *Collinsella*, *Eggerthella, Coriobacterium* and *Slackia* (Yarza et al., 2010). The Family Coriobacteriaceae includes representatives of the high- and low-G+C Gram-positive bacteria (Wade et al., 1999). *Atopobium* spp. have low G+C contents of 39–44 mol%, representing a deep branch within the high G+C Gram-positive Actinobacteria (Stackebrandt et al., 1997). *Atopobium* spp. produce lactic acid, acetic acid and formic acid as end products of fermentation from glucose (Cools et al., 2011). The genera *Slackia*, *Collinsella* and *Eggerthella* have been frequently found in human feces (Nagai et al., 2010, Wade et al., 1999).

The genus *Bifidobacterium* (Phylum Actinobacteria; Family Bifidobacteriaceae) includes high G+C content Gram-positive bacteria, generally strictly anaerobic (some species tolerate moderate oxygen concentrations), non-spore-forming, non-motile, and

non-filamentous polymorphic rod-shaped bacteria. The species *Bifidobacterium catenulatum*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium adolescentis*, *Bifidobacterium pseudolongum*, *Bifidobacterium breve*, *Bifidobacterium angulatum*, *Bifidobacterium dentium, B. bifidum and B. ongum* are commonly found in fecal samples (Mättö et al., 2004).

Although the majority of the microbiota is found within the lumen of the large intestine, microorganisms associated with the mucosa are probably of greater importance to the host (Frank and Pace, 2008). The composition of the mucosa-associated microbiota is uniform along the large intestine (Zoetendal et al., 2002, Eckburg et al., 2005, Lepage et al., 2005, Green et al., 2006) but differs from the fecal microbiota of the same person (Zoetendal et al., 2002, Eckburg et al., 2005), suggesting that the epithelial wall and overlying mucus layer maintain a more stable environment than is present in the lumen (Frank and Pace, 2008). Feces are the most commonly used sample material in human gut microbiota studies due to sampling limitations. However, although fecal samples do not exactly reflect the microbiota composition in the whole GI-tract, most of bacteria leave it via the fecal route and therefore a variation in the fecal microbiota composition reflects a GI-tract related effect (Zoetendal et al., 2001).

1.3 Diet and the large intestinal microbiota

The influence of the diet on the GI microbiota composition and activity has been discussed for several decades (Savage, 1977, Finegold et al., 1983). Early culture-based studies comparing defined diets (e.g. Japanese versus Western) did not show major differences in the composition of the resident fecal microbiota (Finegold et al., 1974), whereas chemically defined diets produced diminished fecal mass associated with compositional changes in the microbiota (Attebery et al., 1972). Advances in molecular microbiological techniques have expanded the knowledge on gut microbial ecology (Zoetendal and Mackie, 2005) and study of the impact of diet and dietary changes on the resident microbiota. At the same time, the diet itself has been changing worldwide as a result of alterations in life-style, agricultural practices and population growth (Kau et al., 2011). Controlled diets, such as those having high protein and reduced carbohydrate content (Russell et al., 2011), or diets differing in non-digestible carbohydrate content (Walker et al., 2011), have been used to study the influence of diet on the microbiota. In addition, fecal microbiota of people having different types of habitual diets (e.g. vegetarians or vegans versus omnivores (Zimmer et al., 2012, Kabeerdoss et al., 2011) or from geographically distinct areas (Lay et al., 2005a, De Filippo et al., 2010) have been characterized. It has become evident that the diet has a considerable effect on the fecal microbiota (Walker et al., 2011). Diet is of primary importance as a source of microorganisms and especially as a substrate for the intestinal microbes (Leser and Molbak, 2009). The main metabolic function of the intestinal microbiota is the fermentation of non-digested dietary materials and endogenous mucus produced by intestinal epithelial cells (Guarner and Malagelada,

2003). Fermentation is the process in which microorganisms break down dietary and other substrates under anaerobic conditions, to obtain energy for growth and maintenance of the cellular functions (Cummings and Englyst, 1987). Fermentation activity differs in the different parts of the large intestine, being the cecum and ascending colon the most metabolically active areas of the proximal intestine.

1.3.1 Dietary nutrients affecting the large intestinal microbiota

1.3.1.1 Carbohydrates

Carbohydrates are organic molecules that contain carbon, hydrogen and oxygen, and are categorized as simple sugars or monosaccharides, oligosaccharides and polysaccharides (Stryer, 2000). Monosaccharides are seldom found free in nature and are typically linked into disaccharide and polysaccharide forms through glycosidic bonds. Oligosaccharides consist of short chains of monosaccharide residues; they are water soluble and often quite sweet (Roberfroid, 1993). When numerous monosaccharides are joined together they form molecules of medium to high molecular weight called polysaccharides, the most abundant carbohydrates found in nature (Nelson, 2000).

Structural cell wall components in plants are primarily cellulose, hemicellulose, pectin and the non-polysaccharide lignin. Whereas pectin and some hemicelluloses, in addition to gums and mucilages, are soluble and readily fermented by the colonic microbiota, other hemicelluloses, lignin and cellulose are insoluble and much less fermentable (Wong et al., 2006). Cellulose constitutes more than 50% of all the carbon derived from plants and is found in their cell walls, particularly in stalks, stems, trunks and all the woody portions of the plant body (Stryer, 2000). Cellulose is a linear polymer of glucose residues linked by (β1→4) glycosidic bonds (Nelson, 2000). The β-configuration allows cellulose to form very long straight chains, resulting in great mechanical strength. Humans cannot utilise cellulose as an energy source because they lack the enzyme cellulase to hydrolyse the (β1→4) linkages. Hemicellulose consists of mixed polymers of sugar units with side units commonly consisting of galactose, arabinose and uronic acid units, usually methylated. Typical types of hemicellulose polysaccharides include xylan, glucomannans and galactans. Pectin substances are usually found in the primary cell walls and intercellular layers of land plants. Some plant tissues are especially rich sources of pectins, for example citrus fruits, apples and sugar beet pulp.

The human absorptive capacity for carbohydrates is limited to only a few of the many possible disaccharide and oligosaccharide configurations in the food supply. In addition, only three monosaccharides, glucose, galactose and fructose, are absorbed in the human intestine. Amylase, which is secreted by the salivary glands and pancreas, cleaves the α-bond of starch polysaccharides (Nelson, 2000). Additionally, enzymes from the brush border of the intestinal mucosal cells hydrolyse glycoside bonds of the disaccharides sucrose, maltose, isomaltose, and lactose. Carbohydrates containing other linkages cannot be digested by human

enzymes, and therefore reach the large intestine available to be fermented by indigenous bacteria. They are commonly classified as dietary fibre. Fibres are the main substrates available to the bacteria in the human colon (Cummings and Macfarlane, 1997a), and their fermentation produces the primary source of energy in the large intestine. A large proportion of these carbohydrates is starch resistant to the activities of host amylases (resistant starch). The remainder of the carbohydrate entering the colon is made up of unabsorbed oligosaccharides such as raffinose, stachyose, fructo-oligosaccharides, galacto-oligosaccharides, polydextrose, pyrodextrins (degradation products of starch), in addition to non-starch polysaccharides (Cummings and Englyst, 1995).

The solubility of dietary fibres that reach the large intestinal lumen is variable. Therefore, carbohydrate polymers with different solubilities are likely to be digested at different rates. The wide variation in the solubility and digestibility of the carbohydrates reaching the colon may affect the species composition of the microbiota along the intestinal tract (Koropatkin et al., 2012). For example, highly soluble carbohydrates might be metabolized more rapidly by bacteria and may be processed in proximal regions of the colon, whereas insoluble fibre or complex polysaccharides may take longer to degrade and thus reach more distal regions. Moreover, the carbohydrate digestibility follows a longitudinal gradient along the colon, reciprocal to the thickness of the intestinal mucus barrier, with greatest thickness in the sigmoid colon and rectum, where mostly insoluble or indigestible carbohydrates are likely to be present (Matsuo et al., 1997). In the proximal colon, the mucous layer is thin, the transit time of colonic content is faster, and bacteria are likely to target more soluble and rapidly digestible carbohydrates. By contrast, the distal colon has a much thicker mucous layer, transit time is slower, and the residual carbohydrates that fuel bacterial growth are likely to be less soluble and therefore take longer to degrade (Koropatkin et al., 2012).

The insoluble carbohydrates, in particular plant cell wall components such as cellulose or resistant starch particles, are decomposed by primary degraders capable of binding and digesting these polysaccharides (Leitch et al., 2007, Flint et al., 2012). It is estimated that up to 70% of cellulose and hemicellulose present in the normal food material is fermented during passage through the large intestine. The bacteria involved include members of both Gram-positive Firmicutes and Gram-negative *Bacteroides* spp. (Flint et al., 2008, Scott et al., 2008). After initial degradation of these complex carbohydrates, more soluble polysaccharides are able to be digested by the secondary degraders (Koropatkin et al., 2012). Solubilisation of the matrix polysaccharides results in cross-feeding to other groups of bacteria, involving fermentation products such as e.g. H_2 and lactate as well as partial degradation products (Flint et al., 2007, Belenguer et al., 2006). Metabolic cross-feeding is a central feature in anaerobic microbial communities. Among the intestinal microbiota, it occurs between primary degraders of complex substrates and other bacterial species that metabolize the first set of products, forming others (Scott et al., 2011).

Carbohydrate fermentation in the colon results in the production of SCFA, mainly butyrate, acetate and propionate (Cummings, 1981), and a number of other

metabolites such as lactate, pyruvate, ethanol and succinate (Blaut and Clavel, 2007). SFCA are the principal aqueous solute in colonic contents, and their concentration in feces can exceed 100 mM. It has been estimated that 90% of the SFCA are absorbed across the intestinal wall (Cummings and Macfarlane, 1997b). The degree to which fibre is metabolized by colonic bacteria and the products of fermentation depends on the specific dietary substrates. High-fibre diets generally increase fecal bulking, SCFA production and the transit rate along the large intestine.

Butyrate is absorbed by the intestinal mucosa where it is the main energy source for colonocytes (Cummings, 1981), providing up to 70% of their requirements (Pryde et al., 2002). When deprived of butyrate, colonocytes undergo autophagy (Donohoe et al., 2011). Butyrate has anti-inflamatory and anti-carcinogenic effects (Perrin et al., 1994, Young et al., 2005). Two important groups of butyrate-producing bacteria are found within the phylum Firmicutes: *E. rectale* and *Roseburia* spp., comprising 5–10% of the total microbiota, and *F. prausnitzii.* Acetate and propionate are absorbed into the blood circulation and utilized by other organs (Jeffery and O'Toole, 2013). Propionate is transported to the liver, where it has a role in glucogenesis, lipogenesis and protein synthesis (Hooper et al., 2002). Genera within the Family Veillonellaceae include propionate-producing bacteria such as *Megasphaera*, *Veillonella*, *Megamonas* and *Selenomonas* (Walker et al., 2005). Acetate is transported to the peripheral tissues via blood circulation, and is a substrate for lipid and cholesterol synthesis (Hooper et al., 2002). In addition, acetate is utilized by resident bacteria of the colon, in particular the butyrate-producing bacteria (Duncan et al., 2004a). Many colonic bacteria produce lactate as a fermentation end product. However, only low levels of lactate are usually detected in feces of healthy individuals (Duncan et al., 2007), since it serves as a substrate for lactate-utilizing bacteria such as *E. allii* and *A. caccae* (Duncan et al., 2004b) and sulphate-reducing bacteria. Up to 20% of butyrate formation is estimated to be derived from lactate (Belenguer et al., 2006). Since many metabolic properties are shared among the microbiota community, it is difficult to link the capacity of producing specific SCFA to phylogenetic information.

Carbohydrate fermentation is enhanced by mechanisms that decrease the volume of colonic gas, involving utilization of H_2 which is formed by many of the anaerobic bacteria inhabiting the colon. H_2 is consumed by methanogens, acetogens and sulphate-reducing bacteria, which convert this gas to methane, acetate or hydrogen sulphide, respectively, depending on the types of microorganisms present (Sahakian et al., 2010). The end product of sulphate reduction, hydrogen sulphide, is highly toxic to the intestinal epithelium and may contribute to colorectal disease (Marquet et al., 2009).

1.3.1.2 Proteins

Proteins are dehydration polymers of amino acids joined by a specific type of covalent bond. Dietary sources make up at least 50% of the protein material that reaches the large intestine every day, while the remaining proteins are produced

endogenously. Dietary proteins undergo structural changes during ingestion, digestion and absorption. The digestibility of proteins is affected by the type of protein and its state of processing before ingestion. Proteins and their hydrolytic products are largely hydrophylic and, unlike fats, do not require bile acids for solubilization (Ahnen, 1995).

Ingested proteins are first hydrolyzed by proteinases such as pepsin, trypsin and chymotrypsin in the small intestine to produce peptides of various lengths. The peptides produced are further digested by brush-border peptidases at the surface of the epithelial cells to amino acids, while some oligopeptides remain unhydrolysed. Peptides are therefore present at different stages of the digestion and may exert a variety of functions in the GI-tract (Shimizu and Son, 2007).

The carbohydrate fermentation mainly occurs in the proximal part of the colon, whereas protein fermentation takes place in the distal colon (Guarner and Malagelada, 2003). As the digesta moves through the distal colon, carbohydrate availability decreases and protein and amino acids become the main bacterial energy source (Macfarlane et al., 1992). Once carbohydrate sources have been used up in the proximal colon, most microorganisms switch to protein fermentation to salvage energy (Ouwehand et al., 2005). The predominant proteolytic species identified in the human large intestine are *Bacteroides* spp. and *Propionibacterium* spp., present at 10^{11}-10^{12} and 10^{8}-10^{10} CFU per g of dry feces, respectively (Macfarlane et al., 1986). Other proteolytic species include the genera *Clostridium*, *Fusobacterium*, *Streptococcus* and *Bacillus.* The *Bacteroides* enterotype has recently been associated with animal protein and saturated fats intake, suggesting that the high meat consumption characterizing the western diet modulates this bacterial enterotype (Wu et al., 2011).

Although proteins provide a less significant energy source in the large intestine, their importance lies mainly in the effects they have on the intermediary metabolism of the host (Hughes et al., 2000). Whereas carbohydrate fermentation leads to perceived health-promoting metabolites, anaerobic degradation of proteins yields toxic metabolites, e.g. sulphur-containing compounds such as ammonia, as well as phenolic and indolic compounds. The fact that protein is a major constituent of meat products and that protein fermentation metabolites such as ammonia, phenolic compounds and tryptophan metabolites have been found to be potentially carcinogenic, suggests a possible relation between meat intake, protein fermentation and colon cancer (Windey et al., 2012). Therefore, the impact of protein fermentation on intestinal health has become particularly relevant nowadays when widespread application of high protein diets for weight loss and body weight management have gained popularity (Windey et al., 2012).

1.3.1.3 Fats

Dietary fats are essential for the digestion, absorption, and transport of fat-soluble vitamins and fat-soluble phytochemicals such as carotenoids and lycopenes (Mahan and Escott-Stump, 2004). Dietary fat slows gastric emptying, depresses

gastric secretions, and stimulates biliary and pancreatic flow, thereby facilitating the digestive process. Fats are composed of fatty acids, i.e. carboxylic acids with hydrocarbon chains, which are classified as saturated (no double-bonds) or unsaturated (Nelson, 2000). Fatty acids are important molecules that play a role as signaling molecules of their own metabolism (Martins dos Santos et al., 2010).

The absorption of fat in the small intestine is generally efficient, although fractions of dietary fat may escape into the feces depending on the amount ingested (Fava et al., 2012). Long chain fatty acids that enter the large intestine are not absorbable by this organ and undergo a series of bacterial modifications (Davidson and Magun, 1995). It has been suggested that the gut microbiota metabolize dietary fats (e.g. by producing diacylglycerols from polyunsaturated fats), convert primary bile acids into secondary bile acids and impact on the enterohepatic circulation of bile acids and fat absorption from the small intestine (Zhang et al., 2009).

Few human studies have investigated the effect of high-fat diets on the fecal microbiota composition. Fecal samples of individuals in a low carbohydrate/high fat diet had lower counts of bifidobacteria than individuals in a high carbohydrate/low fat diet (Brinkworth et al., 2009). On the other hand, obese individuals on a high monounsaturated fat (MUFA) diet with either high or low glycemic index, did not differ in their fecal microbial numbers (Fava et al., 2012). Furthermore, a recent metagenomic study with healthy volunteers found the *Bacteroides* enterotype to be highly associated with the consumption of fat, in particular with saturated fat (SFA) and MUFA (Wu et al., 2011). In contrast, mice fed with high SFA diets have been associated with lower proportions of fecal Bacteroidetes than mice fed with unsaturated fat diets (de Wit et al., 2012, Gibson and Roberfroid, 1995). Mice models are frequently used to understand the role of the intestinal microbiota in obesity, since these animals can be housed under controlled conditions and fed specific controlled diets such as diets rich in fat. Human studies lack these levels of control and thus shifts in the microbiota are considerably more variable (Clarke et al., 2012). A study that compared genetically induced obese mice fed a low-fat diet with wild-type mice fed either a low-fat or high-fat diet observed compositional changes in the fecal microbiota primarily as consequence of the high-fat diet rather than of genetically induced obesity (Murphy et al., 2010). Moreover, administration of a high-fat diet to both wild-type and *RELMβ* knockout mice, resistant to fat-induced obesity, increased the relative proportions of the phyla Proteobacteria, Firmicutes, and Actinobacteria in the feces, whereas the levels of Bacteroidetes decreased in both mice (Hildebrandt et al., 2009). This result indicated that the fat content in the diet itself rather than the obese state of the host induced the changes in the microbiota composition. The reason why the intestinal microbiota changes in response to high-fat diets is still not clear, and the relationships between changes in the microbiota and disease development therefore remain to be elucidated (Yokota et al., 2012).

1.3.1.4 Polyphenols

Polyphenols are regular components of foods, being the most abundant flavonoids in the human diet (Lee et al., 2006). The main dietary sources of polyphenols are fruits, beverages such as coffee, tea and wine, chocolate, and to a lesser extent, vegetables, cereals, and legume seeds (Scalbert et al., 2002). Besides providing colour and flavour to fruits and vegetables, polyphenols influence health as a consequence of their antioxidant and antimicrobial properties, free-radical scavenging activity (Duda-Chodak, 2012), and protective effect against cardiovascular disease, cancer and other degenerative conditions (Guarner, 2008). Although flavonoids and their glycosides can be absorbed through the GI-tract (Kühnau, 1976), their intestinal absorption is usually slow, incomplete, and thus highly variable. Most flavonoids are glycosylated in food, which influences absorption through the intestinal barrier (Scalbert et al., 2002). Unabsorbed dietary phenolics and their metabolites, in addition to their direct beneficial effect on the human tissues, exert significant effects on the intestinal environment by modulation of the microbiota (Dridi et al., 2009, Duda-Chodak, 2012). Tea phenolics (e.g. epicatechin, catechin, gallic acid and caffeic acid) significantly repress certain bacteria such as *Clostridium perfringens* and *Clostridium difficile* and members of the *Bacteroide*s spp., whereas members of bifidobacteria, *Lactobacillus* spp. and non-pathogenic *Clostridium* spp. were relatively unaffected (Lee et al., 2006). Moreover, the consumption of red wine polyphenols increased the numbers of *Enterococcus*, *Prevotella*, *Bacteroides*, *Bifidobacterium*, *Eggerthela lenta* and *B. coccoides-E. rectale* groups in the fecal microbiota of adult man (Sahakian et al., 2010). Knowledge about the impact of polyphenols on the composition and activity of the intestinal microbiota is poor, although recently developed technologies will certainly lead to a better understanding of the interactions between polyphenols and the microbiota.

1.3.2 Diet, obesity and the intestinal microbiota

The incidence of overweight and obesity has increased over recent decades in developed countries. In 2008, the World Health Organization (WHO) estimated that over 1.4 billion adults were overweight and, of these, 200 million men and nearly 300 million women were obese. Moreover, more than 40 million children under the age of five were overweight in 2010 (World Health and Organization, 2012). In adults, overweight and obesity conditions are usually classified according to the body mass index (BMI), defined as an individual's weight in kilograms divided by the square of the height in meters. A BMI value ≥ 25 kg/m^2 corresponds to overweight and a BMI ≥ 30 kg/m^2 corresponds to obesity. BMI provides a useful population-level estimation of overweight and obesity in adults. However, it is the same for both sexes and ages and so it should be considered as an approximate guide since it may not reflect the same degree of fatness in different individuals (World Health and Organization, 2012).

The balance between energy intake and energy expenditure is the basis of weight management throughout life (Lawson et al., 2004). Energy intake is regulated by several mechanisms including hormones, body-fat storages, gut peptides and post ingestion factors (Frary and Johnson, 2004). On the other hand, energy expenditure is explained by the resting metabolic rate, the thermogenic effect of food and voluntary physical activity. A longstanding positive energy balance results in overweight and obesity. Although abnormalities in any of the regulation mechanisms can lead to weight fluctuations, evidence strongly suggests that dietary and activity patterns are the primary causes of the weight gain in industrial societies (Willett, 1998). Evidence is provided e.g. by the dramatic changes in the prevalence of overweight in individuals migrating from countries with low adiposity to industrialized countries (Willett, 1998), where the consumption of energy-dense foods rich in fat, salt and sugars has been increasing. In parallel, there has been a decrease of physical activity as a consequence of the sedentary lifestyle, changing modes of transportation and urbanization. Since obesity is a risk factor for cardiovascular diseases, diabetes mellitus, liver and gall bladder disease, and is associated with an increased risk of mortality (Ogden et al., 2007), the obesity epidemic has induced new studies aiming at identifying environmental factors that could play a role in the energy balance.

The development of obesity has recently been associated with the gut microbiota composition, in particular with the increased capacity of the microbiota to harvest energy from the diet (Turnbaugh et al., 2006). The finding that germ-free mice were apparently protected against diet-induced obesity (Bäckhed et al., 2004) has however been contradicted (Fleissner et al., 2010). These effects were shown to be highly dependent on the type of high-fat diet fed to the germ-free mice, and were also found to be linked to differences in energy expenditure. Studies on energy balance and energy-restricted diets administered to overweight and obese individuals have associated the caloric and nutrient intake with the fecal microbiota composition (Angelakis et al., 2012). The weight loss in a group of 12 obese individuals assigned to either a fat-restricted or a carbohydrate-restricted diet during the course of one year was associated with an increase in the relative abundance of Bacteroidetes, while that of Firmicutes decreased (Ley et al., 2006b). Moreover, a 10-week energy-restricted diet in adolescents brought about increased counts of *B. fragilis* and decreased counts of *Clostridium coccoides* and *B. ongum* in their fecal samples (Santacruz et al., 2009). Similarly, obese adolescents with weight loss above 4 kg had reduced counts of bacteria within the *E. rectale-C. coccoides* group and increased numbers of the *Bacteroides/Prevotella* group, after a 10-week energy-restricted diet (Nadal et al., 2009).

Diet modification with an energy restriction plan is the most common treatment for moderate obesity. However, most people are unable to make the lifelong dietary changes needed for weight management (Bäckhed et al., 2004), thus yielding limited and transient weight loss. Weight loss programs using very low energy diets (VLED) combined with exercise and behavioural changes have provided an alternative treatment for severely obese patients, by producing greater weight loss than the conventional diet and avoiding the excess loss of lean body mass

(Pekkarinen, 1999). VLED are defined as diets providing a maximum of 800 kcal with high quality protein (Mustajoki and Pekkarinen, 2001). Their major advantage is the rapid weight loss. No studies on the gut microbiota of individuals in a VLED plan have hitherto been published.

Bariatric surgery has been increasingly employed in humans as a treatment for severe obesity and has been reported to have an impact on the fecal microbiota composition of obese patients. *Bacteroides*/*Prevotella* group abundance was reported to increase three months after the bypass implantation, being highly dependent on the caloric intake. However, no change in the proportion of Firmicutes/Bacteroidetes ratio was observed in the same study (Furet et al., 2010). A different study observed higher proportions of γ-Proteobacteria, Fusobacteria and *Akkermansia* in the fecal microbiota of gastric bypass individuals, which differed from those of both obese and lean subjects (Zhang et al., 2009). The lean individuals had increased proportions of *Lachnospira* as compared to the obese and gastric bypass individuals. Moreover, the obese individuals of the study had higher numbers of Prevotellaceae and *Methanobacteriales* than the other two groups.

1.3.3 Probiotics and prebiotics: modulation of the microbiota through diet

Prebiotics are defined as a “non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thereby improving the host’s health” (Gibson and Roberfroid, 1995). Different carbohydrates of short chain length have been studied as potential prebiotics, mainly poorly digestible carbohydrates naturally found in foods, such as galacto-oligosaccharides (Davis et al., 2011), xylo-oligosaccharides (Amaretti et al., 2013) and fructans such as inulin and fructo-oligosaccharides (Cummings et al., 2001, Rossi et al., 2005). Inulin-type fructans have been shown to increase populations of *F. prausnitzii* and bifidobacteria in healthy volunteers (Ramirez-Farias et al., 2009, Kolida and Gibson, 2007, Joossens et al., 2011). In addition, oligofructose positively affects the intestinal barrier function, with improvement of the gut permeability and the hepatic and systemic inflammatory tone, by specific changes of the gut microbiota (Everard et al., 2011, Cani, 2012). Other dietary fibres, including whole grain cereals (Costabile et al., 2008), resistant starch, pectins and pectin-oligosaccharides, have also been reported to induce bifidogenic effects. Moreover, human dietary supplementation with fructo-oligosaccharides induced both bifidobacteria and *Collinsella aerofasciens* (Tannock et al., 2004). Prebiotic effects are probably influenced by properties of the substrate such as solubility and branching. Although SCFA production is not a necessary criterion to establish a prebiotic effect, their concentration is often measured since it reflects the rate of fermentation of carbohydrates in the large intestine.

Probiotic bacteria have largely been studied as a means of manipulating the species composition and metabolic activities within the intestinal microbiota, in order to promote health and prevent or manage intestinal disorders (Guarner,

2008). Probiotics are defined as live microorganisms that, when consumed in adequate amounts, confer a health benefit to the host (Salminen et al., 1998). The effects of the probiotics are either direct or indirect, through modulation of the resident microbiota or of the immune system (Marteau et al., 2001). Probiotics should be selected from the intestinal microbiota of humans and should not carry intrinsic resistance to antibiotics (Borriello et al., 2003). Members of the genera *Lactobacillus* and *Bifidobacterium* are the most commonly used probiotics in functional foods and supplements, since they were shown to have a benefitial effect in health (Vitali et al., 2010, Ventura et al., 2009). Fermented foods are the most common vehicles for the delivery of probiotics. *Lactobacilli* have traditionally been included in probiotic products, since these genera are generally regarded as safe. *Lactobacillus* species such as *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus johnsonii*, *L. reuteri*, *L. salivarius* and *Lactobacillus plantarum* have been used as probiotics (Ventura et al., 2009).

Different mechanisms have been proposed to explain how probiotics contribute to human health. Probiotics may promote the competitive exclusion of pathogenic bacteria (Corr et al., 2009), modulate the immune system of the host (O'Flaherty et al., 2010) or enhance the epithelial barrier function. For example, probiotics may increase tight junction function (Anderson et al., 2010) and prevent apoptosis of intestinal epithelial cells (Yan and Polk, 2002, Yan et al., 2007). The intestinal microbiota has been therefore been considered to be a therapeutic target for the development of probiotic functional foods (Sanz et al., 2010, Salminen et al., 1998).

1.4 Adaptation of transient probiotic bacteria to environmental stress

Environmental stress is a common situation faced by all microorganisms in nature (Pichereau et al., 2000). Probiotic bacteria, in particular, are subjected to numerous stressful conditions: during industrial production, food processing, storage, and subsequently during their passage in the GI tract, where they have to resist the unfavourable intestinal conditions and express specific functions under conditions that are unfavourable to growth (Saarela et al., 2004, van de Guchte et al., 2002). Bacterial growth restriction is a consequence of response to different environmental changes or stresses such as pH, temperature (heat, cold), water activity, osmotic pressure, nutrients, oxidation and starvation (De Angelis and Gobbetti, 2004, Somkuti and Steinberg, 1999, Ron, 2006).

Adaptation of microorganisms to adverse environmental conditions typically induces a metabolic stress response. This response consists of changes in the gene expression pattern, with activation or repression of certain genes, leading to the adaptation of cell physiology to new conditions (Aertsen and Michiels, 2004, Pichereau et al., 2000). Like many other microorganisms, probiotic bacteria have developed adaptive mechanisms to respond rapidly to stress, which is essential for their survival in harsh conditions. Adaptive responses of bacterial cultures vary depending on the growth phase (De Angelis and Gobbetti, 2004, Saarela et al.,

2004). Since the exponential growth phase is limited by a number of factors such as nutrient exhaustion and metabolite accumulation, stationary phase is the most common state of bacterial cells in nature. Entry into stationary phase induces a general stress response which prepares cells to survive under different environmental stress conditions and consequently increases bacterial robustness (van de Guchte et al., 2002, Pichereau et al., 2000). Exposure to sub-lethal conditions during growth also develops an adaptive response or tolerance that provides protection against a subsequent exposure to potentially lethal levels of the same stress (Jan et al., 2001, Kim et al., 1999, Collado and Sanz, 2007). Cross-protection to non-homologous stresses can also occur (van de Guchte et al., 2002, Saarela et al., 2004). Cross-protection mechanisms have been observed in different LAB species such as *Lactococcus lactis* (Schmidt and Zink, 2000, Rallu et al., 1996), *B. ongum* and *Bifidobacterium animalis* (Sánchez et al., 2007). Although entry into the stationary phase is also caused by numerous stresses such as cold, heat and pH, adaptation during the stationary phase does not require previous exposure to the stress factor in order to develop the response (De Angelis and Gobbetti, 2004).

As in other bacteria, adaptive responses mediated by molecular chaperones and proteases appear to be one of the major means of stress protection in LAB. However, the molecular basis of such responses to each stress is partially species specific (Sugimoto et al., 2008). The ATP-dependent Clp (caseinolytic) proteins, which include ClpL as a member of the Clp/Hsp100 ATPase family, play an important role during the stress adaptation mechanism (Suokko et al., 2008). Two encoding genes of ClpL proteins, *clpL1* and *clpL2*, have been identified in the strain *Lactobacillus rhamnosus* VTT E-97800 (E800) (Suokko et al., 2005). Suokko *et al.* (2005) also identified *clpL1* in *L. rhamnosus* GG, *L. rhamnosus* ATCC 7469 and *Lactobacillus paracasei* ATCC 25302, and *clpL2* in *L. plantarum* ATCC 14917. According to Northern blot analysis both *clpL* genes were induced in *L. rhamnosus* E800 during heat stress, although *clpL2* to a much lesser extent (Suokko et al., 2005). The genes *clpL1* and *clpL2* have also been detected in another lactic acid bacterium, *Oenococcus oeni*. In *O. oeni* ATCC BAA 1163, *clpL1* expression clearly increased due to acid stress, whereas in the same conditions *clpL2* was induced only slightly (Beltramo et al., 2006).

2. Aims of the study

The overall aim of the study was to evaluate dietary effects on the human fecal microbiota, after optimization of molecular methods for the analysis of microbial groups or species within fecal samples.

The specific aims of the study were:

1. To optimize molecular methods, in particular qPCR methods, for characterization of the human fecal microbiota (Publications I–III).
2. To compare the performance of different commercial fecal DNA-extraction kits in combination with different storage conditions (Publication I and unpublished data).
3. To associate the effect of the habitual dietary intake with the numbers and diversity of the predominant fecal bacterial groups of monozygotic twins (Publication II).
4. To analyse the predominant fecal microbial groups of obese individuals after a very low energy diet and during a follow-up period. In addition, to compare methods for the quantification of fecal samples (Publication III and unpublished).
5. To evaluate the potential of utilizing the information on the expression levels of specific stress response genes (*clpL1* and *clpL2*) in assessing the quality of probiotic products, in addition to the more traditionally used culture-based or viability staining methods (Publication IV).

3. Materials and methods

3.1 Materials

3.1.1 Microorganisms used in this study (Publications I–IV)

The pure cultures used for the optimization and validation of methods performed in the present study are listed in Table 2. The bacterial strains were grown at VTT as recommended by the relevant culture collection. DNA of the Methanobacteriaceae *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* was purchased directly from DSMZ.

Table 2. Microorganisms used for the optimization of group-specific qPCR methods.

Phylum / Species	Family[1]	Strain[2]	Publication
Firmicutes			
Anaerofilum agile	Ruminococcaceae	DSM 4272	I–III
Anaerofilum pentosovorans	Ruminococcaceae	DSM 7168	I–III
Anaerostipes caccae	Lachnospiraceae	VTT E-052773	I–III
Anaerotruncus colihominis	Ruminococcaceae	VTT E-062942	I–III
Blautia coccoides	Lachnospiraceae	VTT E-052778	I–III
Clostridium acetobutyricum	Clostridiaceae	VTT E-93498	I–III
Clostridium beijerinckii	Clostridiaceae	VTT E-93498	I–III
Clostridium bolteae	Lachnospiraceae	VTT E-052776	I–III
Clostridium butyricum	Clostridiaceae	VTT E-97426	I–III
Clostridium clostridioforme	Lachnospiraceae	VTT E-052777	I–III
Clostridium hathawayi	Lachnospiraceae	VTT E-062951	I–III
Clostridium histolyticum	Clostridiaceae	VTT E-052779	I–III
Clostridium indolis	Lachnospiraceae	VTT E-042445	I–III
Clostridium leptum	Ruminococcaceae	VTT E-021850	I–III
Clostridium lituseburense	Peptostreptococcaceae	VTT E-021853	I–III
Clostridium perfringens	Clostridiaceae	VTT E-98861	I–III
Clostridium sporosphaeroides	Ruminococcaceae	VTT E-062947	I–III
Clostridium symbiosum	Lachnospiraceae	VTT E-981051	I–III
Dorea longicatena	Lachnospiraceae	VTT E-052788	I–III
Enterococcus faecalis	Enterococcaceae	VTT E-93203	I–III

Enterococcus faecium	Enterococcaceae	VTT E-93204	I–III
Eubacterium eligens	Lachnospiraceae	VTT E-052844	I–III
Eubacterium hallii	Lachnospiraceae	VTT E-052783	I–III
Eubacterium ramulus	Lachnospiraceae	VTT E-052782	I–III
Eubacterium siraeum	Ruminococcaceae	VTT E-062949	I–III
Faecalibacterium prausnitzii	Ruminococcaceae	DSM 17677	I–III
Lachnospira multipara	Lachnospiraceae	VTT E-052784	I–III
Lactobacillus acidophilus	Lactobacillaceae	VTT E-96276	I–III
Lactobacillus brevis	Lactobacillaceae	VTT E-91458	I–III
Lactobacillus buchneri	Lactobacillaceae	VTT E-93445	II
Lactobacillus casei	Lactobacillaceae	VTT E-85225	I–III
Lactobacillus crispatus	Lactobacillaceae	VTT E-97819	II
Lactobacillus fermentum	Lactobacillaceae	VTT E-93489	II
Lactobacillus gasseri	Lactobacillaceae	VTT E-991245	II
Lactobacillus johnsonii	Lactobacillaceae	VTT E-97851	II
Lactobacillus paracasei	Lactobacillaceae	VTT E-93490	II
Lactobacillus plantarum	Lactobacillaceae	VTT E-79098	I–IV
Lactobacillus reuteri	Lactobacillaceae	VTT E-92142T	I–III
Lactobacillus rhamnosus	Lactobacillaceae	VTT E-97800	I–IV
Lactobacillus rhamnosus	Lactobacillaceae	VTT E-96666	IV
Lactobacillus ruminis	Lactobacillaceae	VTT E-97852	II
Lactobacillus salivarius	Lactobacillaceae	VTT E-97853	I–III
Megasphaera elsdenii	Veillonellaceae	VTT E-84221	I
Roseburia intestinalis	Lachnospiraceae	VTT E-052785	I–III
Ruminococcus obeum	Lachnospiraceae	VTT E- 052772	I
Ruminococcus productus	Lachnospiraceae	VTT E- 052786	I
Subdoligranulum variabile	Ruminococcaceae	VTT E-062950	I–III
Veillonella parvula	Veillonellaceae	VTT E-001737	I–III
Bacteroidetes			
Alistipes finegoldii	Rikenellacea	VTT E-093113	I
Bacteroides caccae	Bacteroidaceae	VTT E-062952	I–III
Bacteroides fragilis	Bacteroidaceae	VTT E-022248	I–III
Bacteroides ovatus	Bacteroidaceae	VTT E-062944	I–III
Bacteroides thetaiotaomicron	Bacteroidaceae	VTT E-001738	I–III
Bacteroides vulgatus	Bacteroidaceae	VTT E-001734	I–III
Prevotella melaninogenica	Prevotellaceae	VTT E-052771	I–III
Parabacteroides distasonis	Porphyromonadaceae	VTT E-062943	I–III
Parabacteroides merdae	Porphyromonadaceae	VTT E-062953	I–III
Actinobacteria			
Atopobium parvulum	Coriobacteriaceae	VTT E-052774	I–III
Collinsella aerofaciens	Coriobacteriaceae	VTT E-052787	I–III
Eggerthella lenta	Coriobacteriaceae	VTT E-001735	I–III
Bifidobacterium adolescentis	Bifidobacteriaceae	VTT E-981074	I–III
Bifidobacterium angulatum	Bifidobacteriaceae	VTT E-001481	I
Bifidobacterium animalis spp. a*nimalis*	Bifidobacteriaceae	VTT E-96663	I
Bifidobacterium animalis spp. *lactis*	Bifidobacteriaceae	VTT E-97847	I
Bifidobacterium breve	Bifidobacteriaceae	VTT E-981075	I

Bifidobacterium catenulatum	Bifidobacteriaceae	VTT E-11764	I
Bifidobacterium longum spp. i*nfantis*	Bifidobacteriaceae	VTT E-97796	I
Bifidobacterium longum spp. l*ongum*	Bifidobacteriaceae	VTT E-96664	I–III
Parascardovia denticolens	Bifidobacteriaceae	VTT E-991434	I
Scardovia inopinatum	Bifidobacteriaceae	VTT E-991435	I
Proteobacteria			
Desulfovibrio desulfuricans spp. *desulfuricans*	Desulfovibrionaceae	VTT E-95573	I–III
Desulfovibrio vulgaris spp. *vulgaris*	Desulfovibrionaceae	VTT E-95573	I–III
Escherichia coli	Enterobacteriaceae	VTT E-94564	I–III
Fusobacteria			
Fusobacterium necrophorum	Fusobacteriaceae	VTT E-001739	I–III
Euryarchaeota			
Methanobrevibacter smithii	Methanobacteriaceae	DSM 861	II
Methanosphaera stadtmanae	Methanobacteriaceae	DSM 3091	II

[1] Phylogenetic affiliation according to NCBI taxonomy

[2] Strains obtained from VTT Culture Collection (http://culturecollection.vtt.fi) and DSMZ German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de)

3.1.2 Collection of human fecal samples (Publications I–III)

The clinical samples collected for different studies are described in Table 3. The main recruiting criterion was absence of recurrent and/or persisting GI symptoms. The exclusion criteria were regular dysbiosis, lactose intolerance, celiac disease, and antimicrobial therapy during the two months prior to the sampling point.

The participants defecated into a plastic container, which was then made anaerobic with a gas generator (Anaerocult A mini, Merck, Darmstadt, Germany). Fresh samples (Publication I) were obtained within 0–4 h from defecation, homogenized, and divided into subsamples in an anaerobic workstation (Don Whitley Scientific Ltd., Shipley, UK). Part of the fresh samples was processed fresh; the remainder was divided into three aliquots for storage under different temperature conditions: one sample was stored at 4°C for 2 days after which it was transferred to -70°C; one at -20°C for one week and thereafter at -70°C; and the third aliquot was transferred directly to -70°C (Publication I and unpublished data). Alternatively, the participants collected the fecal samples at home in a plastic container and stored them in the home freezer before taking the samples to the laboratory where they were transferred to -70°C (Publications II–III).

Table 3. Clinical study material.

Publication	Study	Participants	Sampling	Study approval committee
I	Comparison of storage conditions and DNA-extraction methods of fecal samples	Healthy adults; two subjects: female, 44 years old and male, 51 years old (study of storage conditions and DNA-extraction); 10 subjects: 32–62 years old, 3 males and 7 females (DGGE optimization)[1]	Fecal samples: baseline (two subjects); Fecal samples: baseline, 3 and 6 months (10 subjects)	Ethical committee of VTT Technical Research Centre of Finland, Espoo, Finland
II	Habitual dietary intake and fecal microbiota of monozygotic twins	20 monozygotic twin pairs (40 individuals): 23–32 years old, 11 female pairs, 9 male pairs	Fecal samples: baseline	Ethical committee of the Hospital District of Helsinki and Uusimaa, Finland
III	12 month dietary intervention: 6 week very low energy diet period followed by a energy-restricted diet	16 obese subjects: 20–48 years old, 6 males and 10 females	Fecal samples: baseline, 1.5, 5, 8 and 12 months	Ethical committee of the Hospital District of Helsinki and Uusimaa, Finland

[1] DGGE optimization (10 adults) is not included in the study of this thesis.

3.1.3 Dietary intake (Publications II–III)

Dietary intake was monitored using 3 d food diaries (two weekdays and one weekend day). During the study of the Publication III, food diaries were obtained at the baseline of the study and after the very low energy diet at 5 and 12 months.

Subjects were given clear oral and written instructions by a registered dietician, and were encouraged to maintain their normal eating patterns and to estimate the amounts of all foods and drinks using household measures (Bogl et al., 2011). Food diaries were analyzed with the program DIET32 (Aivo, Finland), which is based on a national database for food composition (National Institute for Health and Welfare, 2009).

3.1.4 Anthropometric parameters (Publications II–III)

In the study of Publication II, weight and height of the subjects were measured in a fasting state wearing light clothes and without shoes. Body fat mass was measured by dual-energy X-ray absorptiometry as described by (Bogl et al., 2011).

In the study of Publication III, anthropometric measurements of weight and height were performed at every study point, whereas body fat mass was measured by dual-energy x-ray absorptiometry at months 0, 5 and 12 as described by (Pietiläinen et al., 2012) (Publication III – Figure 1).

3.2 Methods

3.2.1 Stress treatments applied to *L. rhamnosus* VTT E-97800 (Publication IV)

Acid stress treatments were performed in laboratory and fermenter scale, whereas heat stress was studied only in laboratory scale.

For laboratory scale acid stress tests *L. rhamnosus* E800 cells were grown in 200 ml of General Edible Medium (GEM) (Saarela et al., 2004) at 37°C until the stationary phase) was reached (18 h). Cells were divided into aliquots of 5 ml and centrifuged. For heat stress, cells were resuspended into the same volume of GEM. Triplicates were incubated at 37°C (control), 47°C and 50°C. Samples were collected for viability study and ribonucleic acid (RNA) isolation after 10, 30 and 60 min. For acid stress testing, cells were resuspended in triplicate into 5 ml of GEM adjusted to pH 4.0 or pH 3.5 with 1 M HCl, and incubated at 37°C. Non pH-adjusted GEM (pH 6.0±0.3) was used as a control. Samples were collected for viability determination by culturing on De Man, Rogosa and Sharpe Agar (MRSA), pH measurement, and total RNA isolation after 30, 60 and 180 min incubation.

For the acid stress treatment in fermenter scale, *L. rhamnosus* E800 was grown in 5 l of GEM in a laboratory fermenter (Biostat® CT, B. Braun Biotech International, Melsungen, Germany). Fermentation was performed under controlled temperature of 37°C and pH >5.8 by addition of 25% NH_4OH (Merck, Darmstadt,

Germany), constant stirring of 100 rev/min and under nitrogen flux. After reaching stationary phase (21 h), cells were stressed by addition of 18.5% HCl (Riedel de Haën, Germany) until the pH reached 4.0. Samples were collected for viability determination by culturing on MRSA and total RNA isolation at the end of fermentation (control), and after 30 and 60 min of acid stress treatment.

3.2.2 Freeze-drying (Publication IV)

L. rhamnosus E800 freeze-dried cells were prepared in two batches as previously described (Saarela et al., 2006). Briefly, *L. rhamnosus* E800 was grown in 10 L of GEM for 20 h under pH control, neutralized with NaOH and centrifuged. Cell concentrates were mixed with the carrier polydextrose (10% w/v) and after 1 h incubation at room temperature the cell-carrier pellets were freeze-dried with a standard programme in an Epsilon 2–25 freeze-dryer (Martin Christ, Duingen, Germany). Freeze-dried powders were packed into aluminum foil sachets and stored at -20°C for 24 months. The viability and stability of the powders were studied by culturing on MRSA.

3.2.3 Nucleic acids extraction

3.2.3.1 DNA extraction (Publications I–III)

DNA was extracted using the FastDNA Spin Kit for Soil (QBIOgene, Carlsbad, CA, USA) according to the manufacturer's instructions except that the cells were lysed with a Fast Prep instrument (Bio 101 Savant) at 6.0 m/s for 60 s three times. The extracted DNA was stored at -20°C until analyzed.

In the DNA-extraction experiments (Publication I) the DNA-extraction was performed with the FastDNA Spin Kit for Soil (QBIOgene) using six different protocols differing in the FastPrep lysis step: 1) 60s 4.5 m s^{-1}; 2) 60s 6.5 m s^{-1}; 3) 60s + 30s 6.5 m s^{-1}; 4) 60s + 60s s 6.5 m s^{-1}; 5) 60s + 60s + 30s 6.5 m s^{-1}; 6) 60s + 60s + 60s 6.5 m s^{-1}. In addition, DNA from the samples stored at -70°C was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions using two different protocols: Gram-negative bacteria protocol (lysis at 75°C) and Gram-positive bacteria protocol (lysis at 95°C).

3.2.3.2 RNA extraction (Publication IV)

Total RNA was extracted from the stress-treated *L. rhamnosus* E800 cells and purified by using RNeasy Protect Bacteria Mini Kit (Qiagen, Hilden, Germany) in conjunction with the On-column DNase Digestion with the RNase-Free DNAse Set (Qiagen). One millilitre of each sample from the stress experiments was treated with RNAprotect Bacteria Reagent (Qiagen) according to the manufacturer´s instructions. Freeze-dried cells were first dissolved in 25 ml of peptone saline solution

and diluted 1:10 and 1:100. 1 ml of each sample was treated with RNAprotect Bacteria Reagent (Qiagen) according to the manufacturer's instructions. Cells were frozen at -80˚C until examined.

After thawing the stabilized cells, 350 µl of Buffer RTL with β-mercaptoethanol (10 µl/1 ml RTL) was added per sample. The mixture was vortexed for 5–10 s and transferred to 2 ml Fast-Prep tubes containing acid-washed glass beads (150–212 microns; Sigma, Steinheim, Germany). Cells were lysed in a FastPrep FP120 Instrument for 45 s at 6.5 m/s three times. Samples were centrifuged at maximum speed for 1.5 min and the aqueous upper part was transferred into a new tube to which an equal volume of 70% ethanol was added. Lysates were then transferred to RNeasy Mini spin columns placed in a 2 ml collection tube. The manufacturer's instructions were followed from this point onwards. The concentration and purity of the total RNA were analyzed with a Biophotometer 6131 (Eppendorf, Hamburg, Germany).

3.2.4 Real time PCR (qPCR; Publications I–III)

Optimization and validation of qPCR methods were performed for the amplification of the partial 16S rRNA gene of the microbial group of interest, with the primers listed in Table 4 and the conditions described in Table 5. The qPCR reactions were performed using the High Resolution Melting Master Kit (Roche, Mannheim, Germany) with adjustment of the $MgCl_2$ concentration (Table 5). The qPCR amplifications were carried out using a LightCycler® 480 System (Roche), associated with the LightCycler® 480 Software, Version 1.5 (Roche).

The standard curves were created for each target group by using genomic DNA isolated from pure reference strains (Table 6). The extracted DNA was quantified using a NanoDrop 2000c equipment (Thermo scientific) and standardized. For each microorganism used as a reference, the number of cells present in the volume loaded to the qPCR reaction was calculated on the basis of the genome size and the number of 16S rRNA copies per cell. The 16S rRNA copy numbers were identified through the NCBI Genome database (www.ncbi.nlm.nih.gov). A series of six 10-fold dilutions of the standard DNA was performed per target group and amplified during the qPCR assay.

Table 4. Primers (qPCR, PCR-DGGE) and probes (FISH) used in this study.

Target Probe/primer	Use	Sequence (5´ → 3´)	Reference	Study
Predominant bacteria				
U968-f +GC[1]	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGG		
		GGGCACGGGGGGGAACGCGAAGAACCTTA	(Nubel et al., 1996)	I–III
U1401-f[1]	PCR-DGGE	CGGTGTGTACAAGACCC	(Nubel et al., 1996)	I–III
534R[2]	qPCR	ATTACCGCGGCTGCTGG	(Muyzer et al., 1993)	I–III
358F[2]	qPCR	CCTACGGGAGGCAGCAG	(Muyzer et al., 1993)	I–III
Eub338	FISH	GCTGCCTCCCGTAGGAGTT	(Amann et al., 1990)	III
***Eubacterium rectale* group[3]**				
Ccoc-f	PCR-DGGE	AAATGACGGTACCTGACTAA	(Matsuki et al., 2002)	I–III
Ccoc-r + GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGG	(Maukonen *et al.*, 2006)	
		GCACGGGGGGCTTTGAGTTTCATTCTTGCGAA		I–III
g-Ccoc-F	qPCR	AAATGACGGTACCTGACTAA	(Matsuki et al., 2004b)	I–III
g-Ccoc-R	qPCR	CTTTGAGTTTCATTCTTGCGAA	(Matsuki et al., 2004b)	I–III
Clostridial clusters XIVa+b				
Erec482	FISH	GCTTCTTAGTCAGGTACCG	(Franks et al., 1998)	III
***Roseburia/ E. rectale* group[4]**				
Rrec584	FISH	TCAGACTTGCCG(C/T)ACCGC	(Walker et al., 2005)	III
***Clostridium leptum* group[5]**				
Clept-933 f	PCR-DGGE	GCACAAGCAGTGGAGT	(Matsuki et al., 2004b)	I–III
Clept-1240-r+GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGTC	(Maukonen et al., 2012)	
		GGGGCACGGGGGGGTTTTRTCAACGGCAG		I–III
Clept-f	qPCR	GCACAAGCAGTGGAGT	(Matsuki et al., 2004b)	I–III
Clept-R3	qPCR	CTTCCTCCGTTTTGTCAA	(Matsuki et al., 2004b)	I–III

Ruminococcus bromii/ R. flasvefaciens				
Rbro730	FISH	TAAAGCCCAG(C/T)AGGCCGC	(Harmsen et al., 2002)	III
Rfla729	FISH	AAAGCCCAGTAAGCCGCC	(Harmsen et al., 2002)	III
Faecalibacterium prausnitzii				
Fprau584	FISH	CCTCTGCACTACTCAAGAAAAAC	(Suau et al., 2001)	III
***Bacteroides* spp.**				
Bact596f	PCR-DGGE	TCAGTTGTGAAAGTTTGCG	(Vanhoutte et al., 2004)	I–III
Bacto1080r+GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGGG GGCACGGGGGGGCACTTAAGCCGACACCT	(Maukonen et al., 2012)	I–III
g-Bfra-F	qPCR	ATAGCCTTTCGAAAGRAAGAT	(Matsuki et al., 2004b)	I–III
g-Bfra-R	qPCR	CCAGTATCAACTGCAATTTTA	(Matsuki et al., 2004b)	I–III
***Bacteroides/ Prevotella* group**				
Bac303	FISH	CCAATGTGGGGACCTT	(Manz et al., 1996)	III
Bifidobacteria				
Bif164-f	PCR-DGGE	GGGTGGTAATGCCGGATG	(Satokari et al., 2001)	I–III
Bif662-GC-r	PCR-DGGE	CGCCCGCCGCGCGCGGCGGGCCGGGCGGG GGCACGGGGGGCCACCGTTAGACCGGGAA	(Satokari et al., 2001)	I–III
Bifid-f	qPCR	CTCCTGGAAACGGGTGG	(Matsuki et al., 2002)	I–III
Bifid-r	qPCR	GGTGTTCTTCCCGATATCTACA	(Matsuki et al., 2002)	I–III
Bif164	FISH	CATCCGGCATTACCACCC	(Langendijk et al., 1995)	III
***Atopobium* group**[6]				
Atopo-f	qPCR	GGGTTGAGAGACCGACC	(Matsuki et al., 2004b)	I–III
Atopo-r	qPCR	CGGRGCTTCTTCTGCAGG	(Matsuki et al., 2004b)	I–III
Ato291	FISH	GGTCGGTCTCTCAACCC	(Walker et al., 2005)	III

***Lactobacillus* group**[7]				
Lac1	PCR-DGGE	AGCAGTAGGGAATCTTCCA	(Vanhoutte et al., 2004)	I–III
Lac2-GC	PCR-DGGE	CGCCCGCCGCGCCCCGCGCCCGGCCCGCC GCCCCCGCCCCATTYCACCGCTACACATG	(Vanhoutte et al., 2004)	I–III
Lac1-F	qPCR	AGCAGTAGGGAATCTTCCA		II–III
Lac2-R	qPCR	CATTYCACCGCTACACATG	(Vanhoutte et al., 2004)	II–III
Lab158	FISH	GGTATTAGCA(C/T)CTGTTTCCA	(Franks et al., 1998)	III
Methanogen group				
Met630F	qPCR	GGATTAGATACCCSGGTAGT	(Hook et al., 2009)	II
Met803R	qPCR	GTTGARTCCAATTAAACCGCA	(Hook et al., 2009)	II
Clostridium cluster IX				
Prop853	FISH	ATTGCGTTAACTCCGGCAC	(Hook et al., 2009)	III
Eubacterium hallii				
Ehal1464	FISH	CCAGTTACCGGCTCCACC	(Harmsen et al., 2002)	III
***clpL*1**				
p14	RT-qPCR	TTTCTCGAGTTGCTTTATCAGATGGTTGAGC	(Suokko et al, 2005)	IV
p15	RT-qPCR	TTGGTACCATTATTCTTCGTCCGCCG	(Suokko et al, 2005)	IV
***clpL*2**				
p16	RT-qPCR	TCTCGAGAGGATATTATGGTCACTAAGTTACAC	(Suokko et al, 2005)	IV
p17	RT-qPCR	TTAAGCTTTGCTTTAACTTCCTTTACCAGCTG	(Suokko et al, 2005)	IV

[1] Partial 16S rRNA gene (V_6-V_8 hypervariable region)

[2] Partial 16S rRNA gene (V_3-V_5 hypervariable region)

[3] Clostridial phylogenetic cluster XIVa (Collins et al., 1994)

[4] A subgroup within the Clostridial Cluster XIVa (*E. rectale* group), also detected with Erec482.

[5] Clostridial phylogenetic cluster IV (Collins et al., 1994)

[6] *Atopobium* group comprises of genera such as *Atopobium*, *Eggerthella*, and *Collinsella*

[7] *Lactobacillus* group comprises of genera such as *Lactobacillus, Leuconostoc, Pediococcus*, and *Weissella*

Table 5. Group-specific qPCR amplification conditions.

Target group	$MgCl_2$ (mM)	Real-time PCR amplification conditions			Publications
		Denaturation	Annealing	Elongation	
Predominant bacteria	2.5	95°C, 15 s	50°C, 20 s	72°C, 25 s	I–III
E. rectale group	1.9	95°C, 15 s	64°C, 20 s	72°C, 25 s	I–III
C. leptum group	2.5	95°C, 10 s	60°C, 15 s	72°C, 20 s	I–III
Bacteroides spp.	3.1	95°C, 15 s	58°C, 20 s	72°C, 25 s	I–III
Bifidobacteria	2.5	95°C, 10 s	60°C, 15 s	72°C, 20 s	I–III
Atopobium group	2.5	95°C, 10 s	64°C, 15 s	72°C, 20 s	I–III
Lactobacillus group	3.0	95°C, 15 s	62°C, 20 s	72°C, 25 s	II–III
Methanogen group	2.3	95°C, 10 s	62°C, 15 s	72°C, 20 s	III
clpL 1 gene	3.75	95°C, 10 s	61°C, 10 s	72°C, 20 s	IV
clpL 2 gene	3.0	95°C, 10 s	60°C, 10 s	72°C, 20 s	IV

Table 6. Microbial strains used for the standard curves of each microbial group-specific qPCR assay.

Bacterial group	Species	Strain	Genome size (nt)	16S copy numbers
Predominant bacteria	*Anaerostipes caccae*	VTT E-052773	3 605 636	6
Atopobium group	*Atopobium parvulum*	VTT E-052774	1 543 805	1
Bacteroides spp.	*Bacteroides thetaiotaomicron*	VTT E-001738	6 260 361	5
Bifidobacteria	*Bifidobacterium longum* spp. *longum*	VTT E-96664	2 375 792	4
Clostridium leptum group	*Anaerotruncos colihominis*	VTT E-062942	3 718 888	4
Eubacterium rectale group	*Roseburia intestinalis*	VTT E-052785	4 380 675	1
Lactobacillus group	*Lactobacillus casei*	VTT E-85225	3 079 196	5
Methanogen group	*Methanobrevibacter smithii*	DSM 861	1 727 775	1

3.2.5 Reverse-transcription (RT) and qPCR (Publication IV)

Total RNA was reverse-transcribed to complementary DNA (cDNA) using the Quantitect Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions. The volume of RNA template used per reaction was standardized to 150 h/µl. This volume was optimized within a range of 20 to 300 ng/µl of RNA template by comparison of the PCR amplification curve profiles, crossing points (ct) and emission of fluorescence. Fluorescence emission was monitored in the present study with the reporter SYBR Green I.

qPCR-amplification reactions of the cDNA were performed in the LightCycler® Carousel-Based System (Roche), using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche) and the primers listed in Table 4. The specificity of all the reactions was confirmed by analysis of the melting profile, obtained by dissociation of the DNA after amplification. Data were analyzed using the LightCycler Software 3.5 (Roche). The difference between the average of ct values obtained from the controls and the average of ct values from the stressed samples was calculated and the results compared for each sampling point. At the time 0 min, the difference was considered to be 0 since sample and control cells were obtained from the same batch of growth.

3.2.6 Denaturing Gradient Gel Electrophoresis (DGGE; Publication III)

DGGE analyses of the bacterial group of interest were performed using the primers listed in Table 4 and the conditions described in the Publication I. DGGE profiles were analysed with Bio Numerics software version 5.1 (Applied Maths BVBA). Clustering was performed with the Pearson correlation and the unweighted pair group method by using an optimization of 0.5% and a position tolerance of 1.0%. Bands with a total surface area less than 1% were included in the similarity analysis as previously described (Maukonen et al., 2008).

3.2.7 Fluorescent *in situ* Hybridyzation (FISH; Publication III and unpublished)

Fecal samples were prepared for FISH analysis as previously described (Walker et al., 2005). Briefly, diluted cell suspensions were applied to gelatin-coated slides. Thereafter, the slides were hybridized overnight with 10 µl of the relevant oligonucleotide probes and then washed. Vectashield (Vector Laboratories, Burlingame, CA) was added to each slide to prevent fading. Fluorescing cells were visualized with a Leica DMRXA epifluorescence microscope (Leica, Wetzlar, Germany). The total bacterial numbers were enumerated using the Eub338 probe, whereas specific bacterial groups were assessed using one of the probes listed in Table 4.

3.2.8 Statistical analysis

3.2.8.1 Statistical tests (Publications I, III, IV)

In Publication I, qPCR data was logarithmically transformed in order to be able to use parametric statistical methods. Mean and standard deviation was calculated for each experiment. Student's *t*-test (two-sample assuming equal or unequal variances, depending on the data-set) was used for the statistical analyses of the results.

In the Publication III, paired *t*-tests were used to evaluate the change in the abundance of the bacterial groups studied using FISH and qPCR, between the baseline and 5 month samples.

In Publication IV, the mean and standard deviation were calculated for each experiment. Student's t-test with two tails assuming unequal variances was used for the statistical analysis of the results.

3.2.8.2 Statistical models (Publications II–III)

In Publication II, a linear mixed model was applied to study the effect of BMI and dietary intake of the individuals on the numbers of bacteria obtained by qPCR. The logarithmically transformed number of cells was modeled through linear relationships with the dietary quantitative variable, where intercepts and slopes were assumed to depend on the combination of BMI and the bacterial group. The dietary variables considered were energy intake (kcal/d) and the following macronutrients (g/d): protein, SFA, MUFA, (n-3) polyunsaturated fatty acids (PUFA), (n-6) PUFA, insoluble fibre, and soluble fibre. These variables were considered as fixed effects in the model. Twin individuals and their families were treated as random effects in order to reflect the hierarchical structure of the data and to account for inter-pair (between families) and intra-pair (between co-twins) variation in the data. In addition, the residual variation was assumed to differ across the bacterial groups and a general unstructured residual correlation assumption, which allows any correlation pattern, was included to reflect arbitrary dependences between bacterial groups. The model assumptions were assessed for the initial model as described above. Subsequently, stepwise backwards elimination of non-significant effects was carried out using likelihood ratio tests. For the resulting simplified model pairwise comparisons of estimated mean intercepts and slopes between BMI groups within each bacterial group were carried out with appropriate adjustment of *P* values for multiple testing.

The intra-twin pair difference in dietary intake (the same nutrients as mentioned above) measured between co-twins of the same family, the BMI, and body fat, were related with the intra-pair difference of number of cells per bacterial group, intra-pair difference in diversity and bacterial profile similarities between co-twins. General linear models were fitted for each individual bacterial group. Slopes of the regression lines were assumed to vary according to the concordance/discordance

status of the twin pair for BMI. Backwards stepwise elimination of non-significant effects was carried out using likelihood ratio tests.

The comparison of the group-specific DGGE profiles between the co-twins was performed by calculating a similarity percentage. The intra-pair similarities were divided into intervals for each bacterial group analyzed, and correlated with the intra-pair difference in dietary intake of the macronutrients mentioned above. Similarity groups were labeled as “very low” (0 to 25% similarity), “low” (26 to 50% similarity), “high” (51 to 80% similarity), and “very high” (81 to 100% similarity). In the case of bifidobacteria, no similarity values were obtained above 80%. Mean differences between groups were evaluated by ANOVA.

In Publication III, a linear mixed model was applied to study the effect of the energy-restricted diet over the 12 months in the logarithmically transformed number of cells of the microbial groups studied using qPCR. The model accounted for repeated measurements in the same individual. Moreover, the intercepts and slopes were assumed to depend on the combination of the variables time and microbial group, considered in the model as fixed effects. The individuals were treated as random effects, allowing for the variation between and within subjects. Pairwise comparisons of estimated mean intercepts between the different sampling points within each bacterial group were carried out with appropriate adjustment of *P* values for multiple testing.

The statistical environment R (www.r-project.org) (R Development Core and Team, 2011) was used for the statistical analysis, in particular the R extensions packages “nlme” and “multcomp” (Pinheiro et al., 2012). *P*-values below 0.05 were regarded as statistically significant.

4. Results and discussion

4.1 Optimization of group specific qPCR methods for quantification of fecal samples (Publications I–III)

A total of eight qPCR assays were optimized and validated during this study for quantification of the 16S rRNA gene of the major fecal microbial groups: all bacteria (universal), *E. rectale* group, *C. leptum* group, *Bacteroides* spp., bifidobacteria, *Atopobium* group, *Lactobacillus* group and the methanogen group.

According to FISH studies, the *E. rectale* group, *C. leptum* group and *Bacteroides* spp. account together for 50–70% of the human fecal microbiota (Lay et al., 2005a). Lactobacilli and bifidobacteria, although comprising smaller proportions of the fecal microbiota, were included in the study since both of these groups have traditionally been regarded as beneficial to human health. The *Atopobium* group has received less attention in diet-related studies, although it has been shown with hybridization-based studies that the *Atopobium* group comprises 1–5% of the fecal microbiota (Lay et al., 2005a) and that the proportion of actinobacteria in feces may currently be underestimated (Krogius-Kurikka et al., 2009, Salonen et al., 2010).

In the present study, the amount of fecal DNA was standardized for all qPCR reactions. The fluorescent reporter used to detect template amplification during qPCR was the ResoLight dye (Roche). The fluorescent signal was weak during the initial cycles and could not be distinguished from the background. Thereafter the fluorescent signal increased exponentially as the amount of PCR product accumulated, before saturation of the signal. In the performed qPCR amplifications all the reactions saturated, reaching a plateau at the same level of fluorescence.

The efficiency of qPCR amplifications, estimated from the slope of the ct values against concentration, corresponds to the equation $E=10^{-1/slope}$ where 100% efficiency is equal to 2 (Saunders, 2009). Poor optimization of the qPCR reaction conditions can result in low efficiency. In the present study, the qPCR conditions were extensively optimized. The efficiencies of the amplifications obtained varied between 1.7 and 1.9, but were similar for all the samples and standards within the same qPCR experiment.

External standards consisting of a series of 10-fold dilutions of genomic DNA from a reference strain were used for the absolute quantification of each microbial group (Table 6). The reference strain was carefully selected and validated with FISH analysis. The copy number range of the standards used was always within the range expected of the samples to be quantified. At the end of each qPCR amplification, a melting curve analysis was performed by increasing the temperature to 95°C. By obtaining the melting temperatures of each fluorescent signal, it was possible to distinguish between the specific amplification products from the non-specific and negative controls.

4.2 Effect of different storage conditions and DNA-extraction methods in the quantification of fecal microbiota (Publication I and unpublished)

In qPCR approaches using fecal samples, the first step is always the DNA extraction. An efficient method for the recovery of bacterial DNA is essential for successful molecular analysis of this complex microbial community. Therefore, the impact of commonly used commercial DNA-extraction kits (with several modifications) and storage temperatures was evaluated on the most prevalent fecal microbial groups in the study of Publication I and unpublished.

Mechanical DNA-extraction was performed in both fresh samples and stored samples, i.e. 2 days at 4°C, then -70°C; 1 week at -20°C, then -70°C; and at -70°C. The use of different mechanical lysis methods did not affect the DNA yield from fresh samples, whereas with stored samples both the kit and different modifications to the kits caused variations in the DNA yield. The highest DNA yield was achieved with the most rigorous mechanical lysis (Publication I; DNA-extraction protocol 8: Fast Prep 3x60 s; 6.5 m s^{-1}) at all storage temperatures. The storage conditions did not cause significant differences in the DNA yield when the same protocol was applied to the same sample.

The extracted DNA was analysed using group-specific qPCR, in order to evaluate the abundance of the predominant bacteria, i.e. *E. rectale* group, *C. leptum* group, *Bacteroides* spp., bifidobacteria and *Atopobium* group. The highest numbers of most of the studied bacterial groups were detected from fresh samples. The difference in numbers between the stored and the fresh samples of both subjects 1 and 2 is presented in Figure 2.

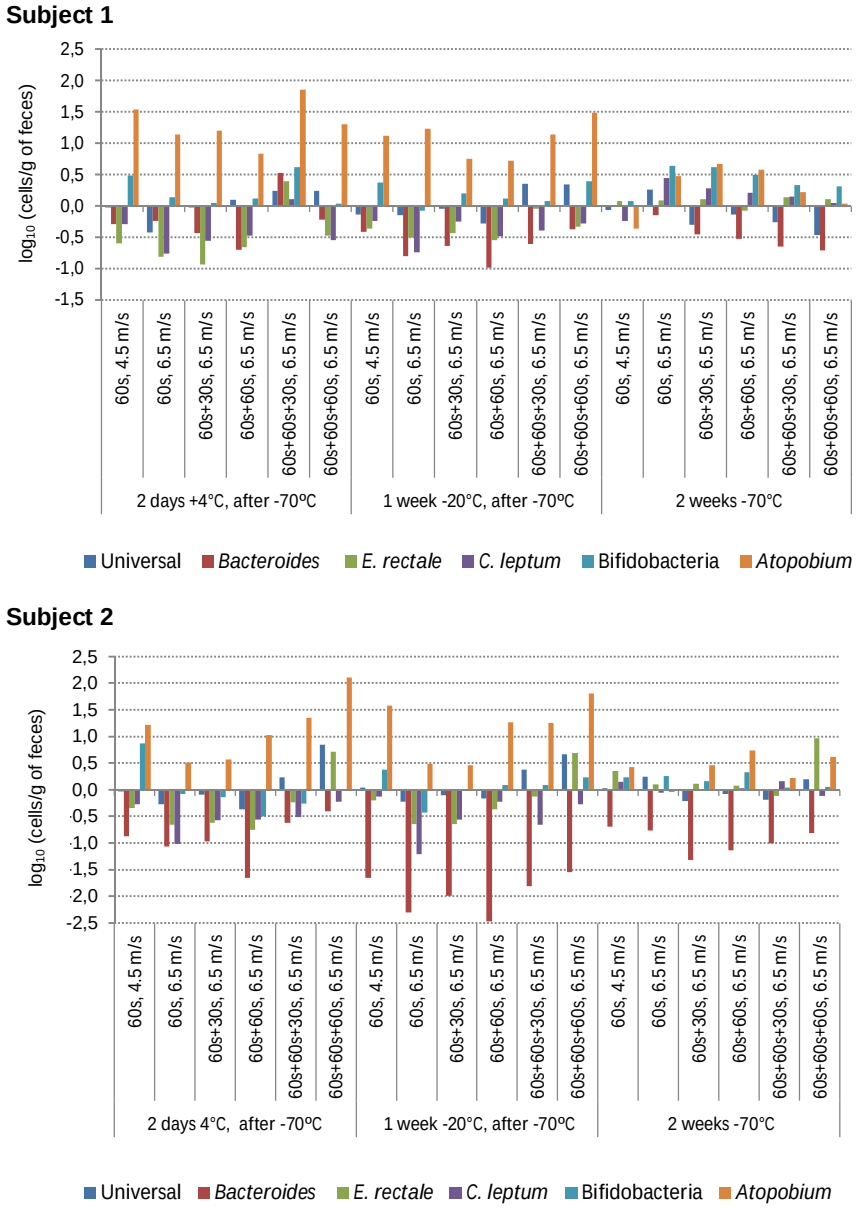

Figure 2. Difference in the number of cells between stored and fresh fecal samples in the subjects 1 and 2, measured using qPCR after several combinations of mechanical DNA-extraction. The results are expressed as log-values (storage-fresh samples, i.e. positive results indicate higher numbers after storage and negative results indicate decrease in the number of cells after storage).

The storage conditions did not have a significant effect on the quantity of universal bacteria, or on bacteria within the *E. rectale* group, *C. leptum* group and bifidobacteria as detected with group-specific qPCR, when similar protocols were compared. By contrast, the storage conditions greatly affected the numbers of the *Bacteroides* spp. The highest numbers of *Bacteroides* spp. measured with qPCR were observed in fresh samples, whereas after one week's storage at -20 ^{o}C the numbers of *Bacteroides* spp. were >1 log lower with all tested protocols as compared to the same sample when fresh. The subject 2, whose number of *Bacteroides* spp. was similar to that of subject 1, had a reduction ranging from 1.6 to 2.5 orders of magnitude after the -20 ^{o}C/-70 ^{o}C storage period (Figure 2). These findings may partly explain why no Bacteroidetes were found in the study of Gill *et al.* (Gill et al., 2006). The numbers of bacteria within the *Atopobium* group were affected by both storage conditions and used DNA-extraction protocols as measured with qPCR. Surprisingly, the highest numbers of *Atopobium* group were detected after an initial storage at 4°C for two days. In addition, the numbers of *Atopobium* group bacteria extracted with the rigorous mechanical DNA-extraction were significantly higher, with more than 1 log difference ($p < 0.05$), after initial storage at -20^{o}C for a week or at 4^{o}C for two days than from fresh samples or from samples stored directly at -70^{o}C (Figure 2). An explanation for this observation may be that the cell wall of *Collinsella* spp. contains a peptidoglycan of type A4β (Kageyama et al., 1999), which may be difficult to lyse without the extra stress of storage at 4°C.

Fecal samples of three additional individuals were analysed using group-specific FISH probes (Figure 3; unpublished data). The number of cells per gram of feces of the bacterial groups analysed with FISH did not significantly vary between the frozen samples, i.e. -20°C and -70°C, and the same samples when fresh. However, the abundance of *Bacteroides* spp. (probe Bac303) tended to decrease when the samples were stored at -20°C. By contrast, the number of bacteria within the *Atopobium* group tended to be higher after storage at -20°C. This observation supports the previous result obtained with qPCR analysis.

The effect of the mechanical DNA-extraction was compared to the enzymatic DNA-extraction in samples stored directly at -70°C using qPCR (Figure 4 and Publication I – Figure 3). The numbers of bacteria within the *Bacteroides* spp. were not significantly affected by the various DNA-extraction protocols, which is in line with previous findings (Salonen et al., 2010).

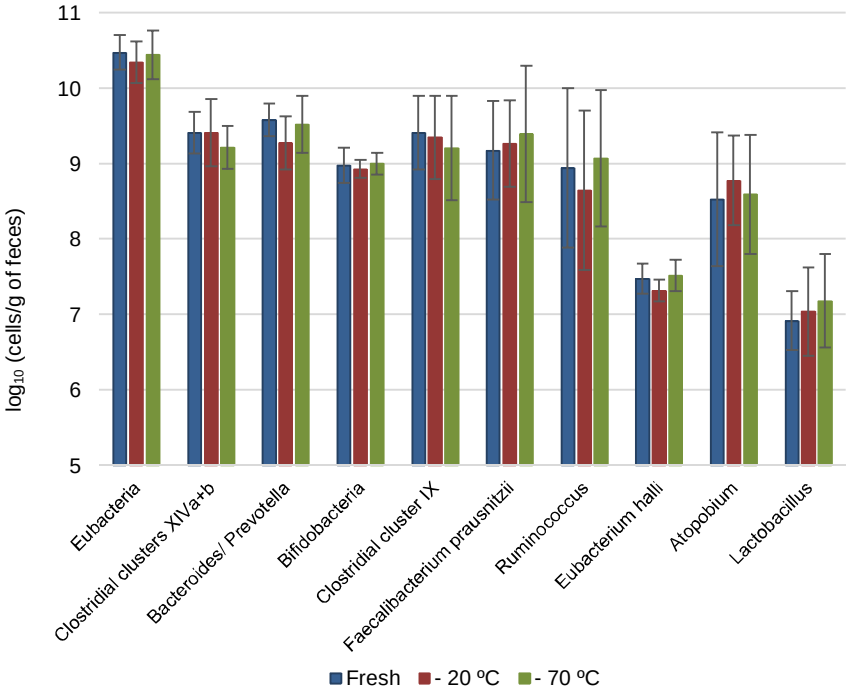

Figure 3. Enumeration of the fecal bacterial groups using group-specific FISH probes, in fecal samples from the subjects 3, 4, 5.

The abundance of *E. rectale* group bacteria was significantly higher after mechanical DNA-extraction than after enzymatic DNA-extraction, with approximately 2 log units difference, regardless of the protocol ($P < 0.05$). Similarly, the numbers of *C. leptum*-group bacteria were around 1.5 log units higher after mechanical DNA-extraction than after enzymatic DNA-extraction ($P < 0.05$). Regarding the *Atopobium* group, the numbers of bacteria within this group were 4–5 log units higher when DNA-extraction was performed mechanically than in the same sample stored at -70°C and with which the DNA-extraction was performed enzymatically. These results may explain why in some studies the *Atopobium* group bacteria are not considered to be part of the normal dominant microbiota (Eckburg et al., 2005), whereas in others, especially those conducted with FISH, the *Atopobium* group bacteria have been shown to constitute 1–8% of the total population of the human gut microbiota (Harmsen et al., 2000a, Matsuki et al., 2004b, Lay et al., 2005a, Mueller et al., 2006). More rigorous mechanical disruption has previously been reported to increase the proportion of Actinobacteria (i.e. bifidobacteria and *Atopobium* group) in the total DNA extracted (Salonen et al., 2010).

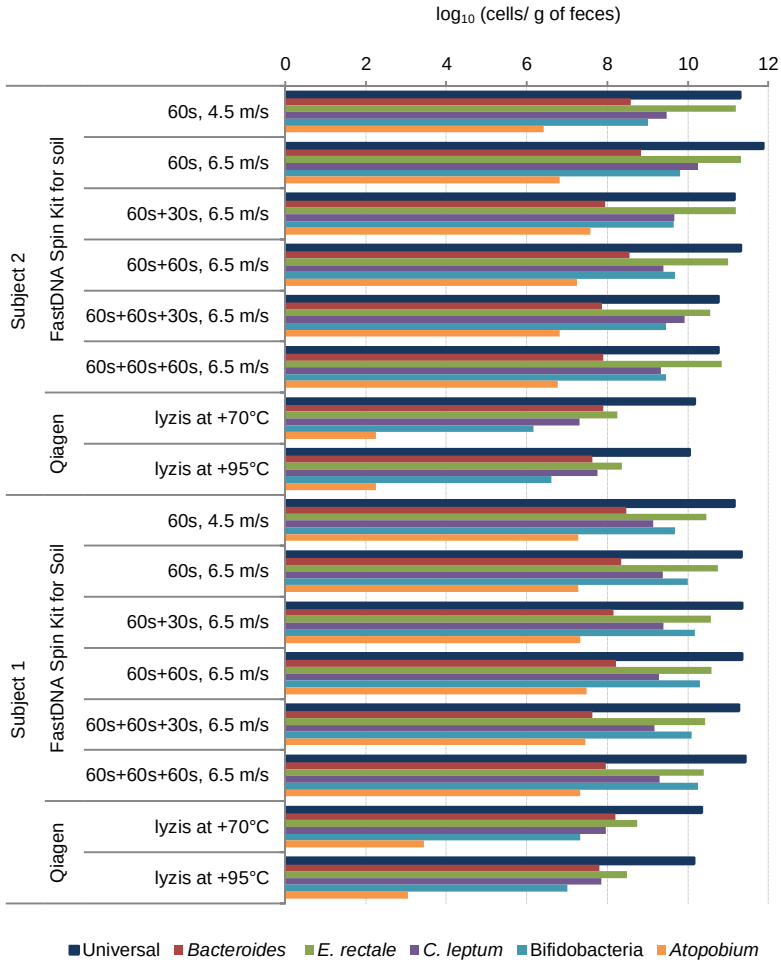

Figure 4. Logarithmically transformed number of cells per gram of feces of the bacterial groups analysed using qPCR, after mechanical DNA-extraction (Fast DNA Spin Kit for Soil) and enzymatic DNA-extraction (Qiagen) of samples stored at -70°C.

The abundance of bifidobacteria was approximately 3 log units higher after mechanical DNA-extraction than after enzymatic DNA-extraction in samples stored at -70°C, as detected with qPCR ($P < 0.05$). Recent contradictory findings have involved the bifidobacterial populations of baby feces. Earlier culture-based studies found the bifidobacterial populations to constitute a dominant part of baby feces (Bullen et al., 1976, Stark and Lee, 1982). Recently, with the available molecular techniques, the results have been partly contradictory. Whereas numerous studies

have reported bifidobacteria to dominate the baby fecal microbiota (Favier et al., 2002, Harmsen et al., 2000b, Magne et al., 2006, Fallani et al., 2010), others have found bifidobacteria to constitute only a minor part of the infant microbiota (Palmer et al., 2007). In those molecular studies in which bifidobacteria predominated in the baby feces, mechanical DNA-extraction has been applied (Favier et al., 2002, Magne et al., 2006) or the samples have been analysed with FISH (Harmsen et al., 2000b, Fallani et al., 2010). On the other hand, in those studies reporting bifidobacteria as only a small proportion of the baby fecal microbiota, enzymatic DNA-extraction using the same commercial kit as in Publication I has been applied (Palmer et al., 2007). Since in Publication I the number of bifidobacteria after enzymatic DNA-extraction was even 3 log units lower than with rigorous mechanical DNA-extraction, differences in DNA-extraction probably explain these contradictory results. Furthermore, another study observed that when enzymatic DNA-extraction was applied the bifidobacterial abundance measured with qPCR was 0.1–1.7%, whereas when FISH was applied to the same samples the bifidobacterial abundance increased to 20.7–83.5% in baby feces (Nakamura et al., 2009).

4.3 Effect of dietary intake on the fecal microbiota of monozygotic twins (Publication II)

The impact of diet on fecal microbiota has usually been assessed by subjecting a group of individuals to the same controlled diet and monitoring shifts in the microbiota. The study of Publication II assessed the effect of the habitual dietary intake on the numbers of fecal microbiota of a population of Finnish MZ twins, through a hierarchic linear mixed model accounting for inter-twin pair and intra-pair variations. The numbers of bacteria measured using group-specific qPCR did not differ between BMI groups (Publication II – Supplementary Figure 1 and Supplemental Table 3). Similarly, the diversity of the studied bacterial groups, defined as the number of the bands obtained by different group specific PCR-DGGE, did not differ between BMI groups (Publication II – Supplemental Figure 2). A recent longitudinal study with obese and lean twins reported a higher proportion of bacteria within the phylum Actinobacteria and a lower proportion of the phylum Bacteroidetes in the obese twins compared to the lean ones, whereas no significant difference in the members of the phylum Firmicutes was observed (Turnbaugh et al., 2009a). Although in the present study no difference in *Bacteroides* spp. numbers was detected between BMI categories, the abundance of this bacterial group significantly decreased when the total energy intake increased ($P = 0.007$), whereas bifidobacteria slightly increased ($P = 0.02$; Publication II – Figure 1A). Furthermore, the co-twins with similar daily energetic intake had more similar *Bacteroides* spp. numbers ($P = 0.03$, $R^2 = 0.3$) and DGGE-profile diversities ($P = 0.02$, $R^2 = 0.3$), as compared to the twin pairs with different energy intakes.

The results of the study of Publication II are in accordance with previous studies which also found a correlation between a low proportion of *Bacteroides- Prevotella* and high energy intake, rather than obesity (Furet et al., 2010). Moreover, according

to Hildebrandt *et al.*, administration of a high-fat diet to both wild-type and *RELMβ* knockout mice, resistant to fat-induced obesity, increased the relative proportions of the phyla Proteobacteria, Firmicutes, and Actinobacteria in the feces, whereas the levels of Bacteroidetes decreased in both mice (Hildebrandt et al., 2009). This result indicated that the fat content in the diet itself, rather than the obese state of the host, induced the changes in the microbiota composition. Although BMI is a validated measure of the nutritional status, other physiological, metabolic and genetic factors, in addition to inadequate physical activity, are behind the etiology of weight balance disorders. Therefore, studies on the relationship between the gut microbiota and host health should not rely solely on the BMI values, but should also consider other variables such as diet composition.

Naturally occurring fats are mixtures of SFA, MUFA, and PUFA, with one predominating type in most foods. Therefore, the intake of different types of fats correlated with the fecal microbiota composition. The greater MUFA consumption was associated with lower numbers of bifidobacteria ($P = 0.0005$) (Publication II – Figure 1B). In addition, co-twins with the same SFA intake had very similar *Bacteroides* spp. profiles (80 to 100% similarity), significantly different from the twin-pairs having distinct SFA intake (0 to 25% similarity; $P = 0.003$) (Publication II – Figure 2A), suggesting that the intake of SFA affects the diversity of *Bacteroides* spp. by targeting specific strains within the same group. In a recent metagenomic study with healthy volunteers, the *Bacteroides* enterotype was found to be highly associated with the consumption of fat, in particular with MUFA and SFA (Wu et al., 2011). These observations suggest that the consumption of fat and animal-derived products, typical of the Western diet, is associated with increased *Bacteroides* spp. prevalence in the human gut microbiota. There are only a few studies in which the correlations between the types of dietary fat and the fecal bacterial composition have been investigated. In the study of Publication II, the increased ingestion of (n-3) PUFA had a significant positive association with the numbers of bacteria within the *Lactobacillus* group ($P = 0.02$) (Publication II – Figure 1C). The increase of fecal *Lactobacillus* group bacterial numbers after (n-3) PUFA intake was also previously reported in a mouse study (Pachikian et al., 2011). Furthermore, in a human study of Santacruz *et al.*, the numbers of lactobacilli remained at the same level even though the ingested amount of total PUFA was greatly reduced (Santacruz et al., 2009). The increase in (n-3) PUFA has been reported to be effective in supporting epithelial barrier integrity by improving trans-epithelial resistance and reducing IL-4 mediated permeability (Willemsen et al., 2008). In addition, several lactobacilli have been shown to enhance the intestinal barrier function (Anderson et al., 2010, Donato et al., 2010). Greater (n-6) PUFA intake was negatively correlated with the numbers of bifidobacteria ($P = 0.003$) (Publication II – Figure 1D). This result is in agreement with previous studies reporting that high (n-6) PUFA intakes decrease certain immune functions, such as antigen presentation, adhesion molecule expression, pro-inflammatory cytokine, and Th1–Th2 responses (Harbige, 2003). Furthermore, genomic DNA of some bifidobacterial strains has been shown to stimulate the production of the Th1 and pro-inflammatory cytokines, interferon (IFN)-γ and TNF-α (Medina et al., 2007). Over-

all, these results indicate an association between dietary fat types and their distinct effect on the fecal microbiota. As a consequence, it appears that balanced diet with regard to fat consumption is critical not only for the host´s health but also for the gut microbiota.

The intake of soluble fibre had a positive association with the *Bacteroides* spp. numbers (*P* = 0.009) (Publication II – Figure 1E). In addition, the levels of *C. leptum* and *E. rectale* groups of bacteria were increased in the individuals that consumed high quantities of soluble fibre. Bacteria within the *Bacteroides* spp. have been recognized as versatile members of the dominant microbiota, carrying a vast array of polysaccharide-hydrolyzing enzymes. Moreover, *Bacteroides* spp. have been associated with the breakdown of soluble fibres (Flint et al., 2008). It has been reported in several studies that high consumption of fibre is associated with an increased proportion of butyrate-producing bacteria (Benus et al., 2010, Louis and Flint, 2007). Since most of the butyrate-producing bacteria belong to the *C. leptum* and *E. rectale* groups, these results are in agreement with the previous studies (Hold et al., 2003, Louis et al., 2007). The group of co-twins who daily consumed the same amount of fibre had very low similarity within Bifidobacteria (0 to 25%), significantly different from the group with high similarity (56 to 80%) (P = 0.008) (Publication II – Figure 2B), suggesting that the type of fibre rather than the amount is more selective when targeting specific bacterial species. For the other bacterial groups studied using DGGE, no significant associations were obtained regarding the dietary intake. No relation was found between the intra-pair DGGE-profile similarities and intra-pair difference of BMI, or body fat. Concordance of co-twins for BMI did not relate to the bacterial diversity or similarity.

4.4 Impact of a very low energy diet on the fecal microbiota of obese individuals (Publication III)

In the study of Publication III, the main fecal bacterial groups of 16 obese individuals (BMI > 30 kg/m^2) were quantified using qPCR during a 12-month energy-restricted diet, which included an initial VLED period of 6 weeks high in protein and low in carbohydrates and fat. In addition, fecal samples from a subset of 8 individuals were analysed using FISH at 0 and 5 months of the study (Publication III – Figure 1).

During the VLED period of 6 weeks, the nutritional intake was similar for all the participants and corresponded to the period with the lowest daily energetic intake over the study (800 kcal), resulting in a decrease of body weight and thus BMI (Publication III – Table 1). The total energy intake decreased on average by 64.6% per day [51.8% (min); 78.3% (max)]. The greater the energy reduction from the baseline, the lower were the numbers of bifidobacteria measured at the end of the VLED (*P*-value = 0.02; R^2 = 0.6). Moreover, the numbers of bacteria within the fecal bacterial groups quantified using qPCR tended to decrease at the end of the VLED, except that the numbers of Bacteroides spp. slightly increased (Publication III – Figure 2). The numbers of bifidobacteria showed the most drastic reduction of

approximately 2 orders of magnitude at the end of the VLED as compared to the baseline ($P < 0.001$), followed by the *Lactobacillus* group that decreased about 1 log unit. The low intake of carbohydrates may have reduced the substrates available for bifidobacteria and bacteria within the *Lactobacillus* group in the large intestine. Furthermore, the reduced dietary intake and the consequent lower consumption of dairy products may have lowered the amount of *Lactobacillus* ingested during the weight loss intervention. In addition, the decrease of bifidobacteria and *Lactobacillus* group bacteria during the VLED possibly reflect the low intake of n-3 polyunsaturated fatty acids (n-3 PUFA). A maximum of 0.5 g per day of α-linolenic acid is recommended by the SCOOP task report of the European Commission for VLED programs. The intake of such low amounts of n-3 PUFA has been associated with lower abundance of bifidobacteria and bacteria within and *Lactobacillus* group in the Publication II of this study. The increase of soluble fiber intake has also been associated with higher numbers of *Bacteroides* spp. and lower numbers of bifidobacteria in the Publication II. These results are consistent with the study of the Publication III since the proportion of fiber during the VLED intervention was higher as compared to the baseline. On the other hand, the high content of protein in the VLED may have contributed to the increase of *Bacteroides* spp. bacteria, the predominant proteolytic species identified in the human large intestine (Macfarlane et al., 1986). The increase of *Bacteroides* spp. during energy-restricted diets has been reported previously (Nadal et al., 2009, Santacruz et al., 2009), although in these studies the reduction of total energy intake was not as drastic as during the VLED intervention of the present study. By contrast, longitudinal studies involving severely obese patients subjected to a gastric bypass are more comparable since the gastric bypass results in a drastic reduction of food consumption and thereafter weight loss, in a short period of time. A recent study reported an increase in the numbers of *Bacteroides/Prevotella* group bacteria 3 months after a Roux-en-Y bypass surgery, whereas the numbers of bacteria within the *Bifidobacterium* and *Lactobacillus/Leuconostoc/Pediococcus* groups decreased significantly (Furet et al., 2010). These results are consistent with the study of Publication III.

After the VLED, the participants followed a personalized diet plan adjusted to their personal needs with individual supervision until 5 months into the study, and thereafter without supervision until 12 months. At 5 months, the total energy intake was higher than during the VLED (~ 1500 vs. 800 kcal/day), while BMI decreased and reached its lowest value during the study (30.4 kg/m^2). The return to the conventional diet decreased the daily proportion of protein intake and increased the intake of fat and carbohydrates. At 5 months, the fecal bacterial groups with more drastic changes were again the bifidobacteria and the *Lactobacillus* group, increasing by 1.2 and 0.6 log units, respectively, although not significantly. The changes in numbers of the microbial groups studied using qPCR followed the dietary intake and not the BMI variation.

Fecal samples from a subset of eight individuals were analysed using FISH at 0 and 5 months of the study, and compared with the qPCR results from the same subset of participants (Publication III – Table 2). The relative proportion of the *Roseburia/ E. rectale* group (Rrec584), a subgroup of the Clostridial cluster XIVa,

was significantly lower at 5 months (1.2%) as compared to the baseline (4.7%; $P = 0.046$). The same trend was observed with bifidobacteria (13% at 0 mo. vs. 6.3% at 5 mo.), however not statistically significant ($P = 0.07$). In a previous study, obese individuals undergoing both moderate and low carbohydrate weight loss diets in opposite orders showed a decrease in the numbers of bacteria within the *Roseburia/ E. rectale* group during both diets (Duncan et al., 2008). Moreover, in the same study the proportion of bifidobacteria significantly decreased in subjects following a 4-week weight loss treatment on both of the above-mentioned diets. These results are in accordance with those of the study of Publication III, since the VLED intervention also had a low carbohydrate content.

The relative abundance of the bacterial groups, calculated as percentage of the universal group counts, was however higher when measured with FISH than with qPCR. The bacterial numbers obtained with the universal qPCR primers at the baseline and 5 months (11.8±0.3 and 11.8±0.4 log (cells/g), respectively) were higher than the counts obtained with the universal FISH probe (10.2±0.2 and 10.2±0.4 log (cells/g), respectively). Therefore, the relative abundance of comparable bacterial groups was higher when measured with FISH than when using qPCR (Publication III – Table 2). Furthermore, the relative abundance of bifidobacteria was significantly different between FISH and qPCR measurements in both sampling points. These observations suggest that caution is necessary when considering relative proportions of fecal bacterial groups obtained with different methods. In addition, the use of different primers or probes targeting similar groups of bacteria within the fecal microbiota should be taken into consideration, especially in the case of Bacteroidetes (Hoyles and McCartney, 2009).

The 8 month samples were collected after the summer holidays, during which time the weight loss diet plan was not rigorously followed by the participants and the average weight loss reached a plateau. The abundance of all the bacterial groups tended to increase at 8 months, except for *Bacteroides* spp. which maintained levels similar to the previous sampling point with approximately 9.5 log (cells/g of feces). Similarly, at 12 months the numbers of *Bacteroides* spp. bacteria did not vary, in contrast to the other fecal groups studied which also decreased to numbers similar to those measured at 5 months. At the end of the study, both the weight and the daily energetic intake measured from our volunteers were lower than at the baseline (nine kg and 630 kcal, respectively). Except *Bacteroides* spp., the other microbial groups studied using qPCR also had lower numbers at the end of the study as compared to the baseline. This study confirms that the alteration of the nutrient load induces changes in the gut microbiota abundance, as suggested before (Jumpertz et al., 2011).

The methanogen group was detected in 56% of the study population using qPCR. For each individual having methanogens, the presence of this group was detected in every sampling point, with the exception of one point from one individual, regardless of the weight loss or weight gain. This observation suggests that the presence of methanogens is host-specific, as alluded to before (Gibson et al., 1988). Although the median of methanogens per gram of feces decreased at the end of the VLED and afterwards increased with the return to the conventional diet,

the inter-individual variation among the participants was high. In a previous study, the number of copies of *M. smithii* species was found to be significantly higher in anorectic patients than in lean controls (Armougom et al., 2009). Methanogens increase the efficiency of bacterial fermentation in the colon by utilizing the end product H_2 (Turnbaugh et al., 2006), thereby enhancing the food fermentation in very low energy diets (Armougom et al., 2009).

Bacterial group-specific DGGEs were used to assess the bacterial diversity and temporal stability during the weight loss intervention. The diversity of the universal fecal bacteria, *E. rectale* group and *C. leptum* group remained similar to the baseline diversities of 29±4, 15±3 and 17±4 bands, respectively. In addition, intra-individual temporal similarity of universal bacteria remained stable between the sampling time points, however, the values varied greatly between individuals (e.g. intra-individual similarity between baseline and 5 months: 34–87%). Intra-individual temporal stability of *E. rectale* group and *C. leptum* group tended to be higher between 8 and 12 months than between baseline and 5 months (Publication III – Supplementary Figure 1). Amplification products of *Bacteroides* spp. and bifidobacteria specific PCR were obtained only from 34% and 39% of the samples, respectively, and there were no study subjects for whom all samples amplified. Although the diversity of the bacterial groups studied with PCR-DGGE remained stable throughout the study period, it has been shown previously that bacterial numbers do not necessarily correlate with bacterial diversity (Maukonen et al., 2006) and therefore these results do not contradict each other. It is however surprising that the variation in dietary intake did not affect the bacterial diversity as measured with DGGE, suggesting a strong host effect on the composition of the fecal bacterial population.

4.5 Expression of *clpL1* and *clpL2* genes in *L. rhamosus* VTT E-97800 after exposure to stress conditions and freeze-drying (Publication IV)

Probiotic bacteria are subjected to numerous stressful conditions during food processing, storage and subsequently during their passage through the GI-tract, where they have to resist the adverse intestinal conditions (Saarela et al., 2004). Probiotic cultures are commonly grown to high-cell densities before undergoing a drying process that produces a probiotic powder usually added to specific carriers such as dairy products (Mills et al., 2011). Cell viability studies are not sufficient to predict the quality of probiotic products (Mättö et al., 2006). Mättö et al. (2006), working with *Bifidobacterium animalis* subsp. *lactis* Bb-12 freeze-dried cells, observed a more prominent decrease in cell functionality (in this case tolerance to low pH) than decrease in viability during storage. Therefore, the potential of using information on the expression levels of specific stress response genes in assessing the quality of probiotic products was evaluated in the study of Publication IV, using the *L. rhamnosus* E800 strain.

The stress conditions used in this study were chosen so that they would not reduce the viability as determined by culturing of the probiotic cells. Therefore, no decline in viability was seen for *L. rhamnosus* E800 cells after the heat or acid treatments performed in this study. Furthermore, LAB starters and probiotics are usually harvested in late-log or early stationary growth phase to achieve maximum cell yield and viability during downstream processing (Saarela et al., 2004).

The expression of *clpL1* and *clpL2* was evaluated in *L. rhamnosus* E800 stationary phase cells by comparing crossing point values obtained from RT-qPCR of samples subjected to different sub-lethal conditions that probiotics do normally encounter during processing, or to freeze-drying. RT-qPCR permits the detection and quantification of mRNA, particularly for low abundant templates (Bustin, 2002). RT-qPCR is a complex assay and the reaction should be carefully optimised. Reverse transcription is a critical step for sensitive and accurate results, since the amount of cDNA produced must reflect the input amount of the mRNA (Kubista et al., 2006). In the present study, the amount of RNA template was standardized to 150 ng/µl. This value was selected since it resulted in good PCR amplification curve profiles during optimization, with lower crossing point values and higher fluorescence. Fluorescence emission was monitored in the present study with the reporter SYBR Green I, which signal is directly proportional to the increase of the double-stranded amplified DNA (Bustin, 2000). Consequently, the lower the crossing point, the more abundant the initial target. Melting curve analysis confirmed the specificity of PCR products from all the stressed samples.

The expression of *clpL1* in *L. rhamnosus* E800 was induced by sub-lethal heat treatments. At 50°C *clpL1* was more pronouncedly expressed when compared to 47°C, especially after a 60 min exposure (Figure 1 – Study IV). At 47°C the expression of *clpL1* showed a slight decrease between 10 min and 30 min of exposure, being constant after 30 min until the end of the treatment with a difference of approximately 2 cycles to the control. The same trend was observed at 50°C during the first 30 min of exposure. However, an increase in the expression was observed 30 min later, with a difference of 5.7 cycles to the control after 60 min. Results were confirmed in two additional tests with 60 min samples (data not shown). In contrast, *clpL2* appeared not to be affected by heat treatments at 47°C or at 50°C in *L. rhamnosus* E800. Northern blot analysis carried out by Suokko *et al*. (2005) revealed that *clpL1* expression was strongly induced in *L. rhamnosus* E800 exponential phase cells subjected to 50°C compared to *clpL2* transcripts, which were only very moderately induced (Suokko et al., 2005). The results of the study IV confirm those of Suokko *et al*. (2005) indicating that the *clpL1* gene is involved in the adaptation mechanism of *L. rhamnosus* E800 cells exposed to sub-lethal heat stress conditions. However, the failure to repeat the anticipated results on the low level of *clpL2* induction can probably be explained by methodological differences in the two studies and also by the differences in the physiology of the cells (stationary phase cells versus exponential phase cells). Induced thermotolerance allows bacteria to survive at otherwise lethal temperatures if briefly pre-exposed to non-lethal heat treatments. In *L. gasseri*, *clpL* is not essential for growth under mild and sub-lethal conditions, but it plays a role in both constitutive

and induced thermotolerance (Suokko et al., 2008). In *Staphylococcus aureus clpL* is also required for the development of induced thermotolerance (Frees et al., 2004). *clpL* disruption in *Streptococcus thermophilus* reduces tolerance to heat and cold shock (Varcamonti et al., 2006) and a ClpL-deficient derivative of *Streptococcus pneumoniae* grew more slowly than the wild-type at high temperature (Kwon et al., 2003). The results of our study confirm those of Suokko *et al.* (2005) indicating that the *clpL1* gene is involved in the adaptation mechanism of *L. rhamnosus* E800 cells exposed to sub-lethal heat stress conditions.

Acid treatments in stationary phase *L. rhamnosus* E800 cells did not induce the expression of either *clpL1* or *clpL2* after moderate stress conditions (pH 4.0 or pH 3.5 for up to 3 h). Possibly the moderate acid treatments used in this study were not harsh enough to induce a measurable expression of *clpL1* or *clpL2* in *L. rhamnosus* E800. Furthermore, the stress treatment procedures used in previous studies and the physiological state of the cells differed from those in our study. In *Oenococcus oeni* ATCC BAA 1163, growth in a special medium at pH 3.5 induced the expression of *clpL1* about twelve-fold and *clpL2* four- to six-fold when mRNA levels were measured by RT-qPCR (Beltramo et al., 2006). Furthermore, late exponential phase cells of *L. reuteri* ATCC 55730 showed induction of *clpL* after a transfer from pH 5.1 to pH 2.7. In another study, stress treatment of growing *Lactobacillus bulgaricus* ATCC11842 cells (pH 3.8, 35 min at 42°C) clearly resulted in induction of ClpL, although transcriptional analysis revealed no induction (Fernandez et al., 2008).

In freeze-dried samples no amplification was observed for *clpL1* and *clpL2* transcripts, which was confirmed by the melting curve analysis. Repeated attempts to isolate good quality or -quantity mRNA from freeze-dried *L. rhamnosus* E800 powders were unsuccessful. The quality of the powders was good (culture-based viability was $1x10^{11}$cfu/g), and therefore loss of viability does not explain this result. Although culture-based analysis indicated that the viability of the freeze-dried samples was high, mRNA from *L. rhamnosus* E800 powders could not be amplified with either *clpL1* or *clpL2* primers. This probably reflects the difficulty in isolating RNA from freeze-dried prokaryotic cell material. Previously RT-qPCR has been successfully applied in the gene expression analysis of dried yeast preparations (Vaudano et al., 2009). In addition, the carrier polydextrose used in the freeze-drying might have interfered with the mRNA isolation or PCR reaction, inhibiting the amplification. To our knowledge this was the first published attempt to isolate mRNA from freeze-dried bacterial cells.

5. Conclusions

In order to quantify the most abundant microbial groups within the fecal microbiota, 16S rRNA gene-based qPCR methods were developed during this study. Group-specific qPCR provided a precise, reproducible and rapid quantification of the amount of PCR products in each amplification cycle through the measurement of fluorescent signal intensity. However, species-specific protocols should be extensively optimized, and validated after the selection of the reference strain for the quantification because the different species within each phylogenetic group have different 16S rRNA gene copy numbers.

Different storage conditions and DNA-extraction protocols affect the numbers of the most abundant bacterial groups in fecal samples as measured with qPCR. In particular, rigorous mechanical lysis of human fecal samples led to the detection of higher bacterial numbers than enzymatic DNA-extraction, particularly in the case of Actinobacteria.

The present study confirms that the dietary and energetic intake modulates bacterial groups within the fecal microbiota. In particular, different types of dietary fat have distinct effects on the fecal microbiota composition, suggesting that a balanced diet with regard to fat consumption is critical not only for the host's health but also for the gut microbiota. In addition, dietary intake within monozygotic twin pairs may be more influential than body fat levels in determining the fecal microbiota. Moreover, during the 12 month weight loss intervention of this study, the change in fecal microbial numbers correlated with the energy intake and not with the change in weight. Therefore, studies on the relationship between gut microbiota and health of the host should not rely only on BMI values but should also consider other variables such as diet composition. Diets with very low energy content affect the major fecal microbial groups, in particular the numbers of bifidobacteria, within a period of 6 weeks. During the same period, the presence of methanogens in the fecal microbiota was not influenced even by drastic changes in the energetic intake. In the end of the 12-mo. intervention, the microbial groups studied had similar numbers to those measured at 5 mo. reflecting that the change in fecal microbial numbers are associated with the dietary intake rather than the body weight variations.

Finally, the induction of clpL genes in *Lactobacillus rhamnosus* VTT-E97800 appears to be detectable with RT-qPCR only in cases when the heat or acid stress is rather extreme. Therefore, although an induction of *clpL1* was observed during the heat stress conditions applied, the expression of this gene cannot be considered to be a good candidate as a marker to study the quality of *L. rhamnosus* products.

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Title	**Dietary effects on human fecal microbiota**
Author(s)	Catarina Simões
Abstract	The establishment of microbial populations in the gastrointestinal (GI)-tract is a complex process, involving microbial and host interactions eventually resulting in a dense and stable population. Recently, the identification of microbial species from fecal samples has become more accurate with the use of 16S RNA gene-based methods. However, although these molecular-based detection methods have apparent benefits over culture-based techniques, they involve potential pitfalls that should be taken into consideration when studying the fecal microbiota, such as the storage conditions and deoxyribonucleic acid (DNA)-extraction. Therefore, the effects of different storage conditions and DNA-extraction protocols on fecal samples were evaluated in this study. Whereas the DNA-extraction protocol did not affect the numbers of *Bacteroides* spp., the abundance of this group showed a significant decrease after one week's storage at -20°C. Furthermore, the numbers of predominant bacteria, *Eubacterium rectale* group, *Clostridium leptum* group, bifidobacteria and *Atopobium* group, were significantly higher in samples stored at -70°C after mechanical DNA-extraction than after enzymatic DNA-extraction as detected with real-time PCR (qPCR). These results indicate that rigorous mechanical lysis leads to the detection of higher bacterial numbers from human fecal samples than enzymatic DNA-extraction. Therefore, the use of different DNA-extraction protocols may partly explain contradictory results reported in previous studies. The composition of the human intestinal microbiota is influenced by host-specific factors such as age, genetics and physical and chemical conditions encountered in the GI-tract. On the other hand, it is modulated by environmental factors with impact on the host during the lifespan, such as diet. The impact of diet on the gut microbiota has usually been assessed by subjecting people to the same controlled diet, and thereafter following the shifts in the microbiota. In the present study, the habitual dietary intake of monozygotic twins was associated with the fecal microbiota composition, which was analysed using qPCR and Denaturing Gradient Gel Electrophoresis (DGGE). The effect of diet on the numbers of the bacteria was described using a hierarchical linear mixed model that included the twin individuals, stratified by body mass index, and their families as random effects. The abundance and diversity of the bacterial groups studied did not differ between normal weight, overweight, and obese individuals with the techniques used. However, intakes of energy, monounsaturated fat, (n-3) polyunsaturated fat, (n-6) polyunsaturated fat and soluble fibre had significant associations with the fecal bacterial numbers. In addition, co-twins with identical energy intakes had more similar numbers and DGGE-profile diversities of *Bacteroides* spp. than co-twins with different intakes. Moreover, co-twins who ingested the same amounts of saturated fat had very similar DGGE-profiles of *Bacteroides* spp., whereas co-twins with similar consumption of fibre had very low bifidobacterial DGGE-profile similarity. Thereafter, the impact of the energy intake on the fecal microbiota of a group of 16 obese individuals was assessed during a 12 month intervention, which consisted of a 6 week very low energy diet (VLED) and thereafter a follow-up period of 5, 8 and 12 months. The diet plan was combined with exercise and lifestyle counselling. Fecal samples were analyzed using qPCR, DGGE and fluorescent *in situ* hybridization. The effect of the energy restricted diet on the fecal bacterial numbers was described using a linear mixed model that accounted for repeated measurements in the same individual. The VLED period affected the major fecal microbial groups; in particular bifidobacteria decreased compared to the baseline numbers. Furthermore, the change in numbers of the fecal bacterial groups studied, with the exception of *Bacteroides* spp., followed the energy intake and not the weight changes during the 12 months. Methanogens were detected in 56% of the participants at every sampling time point, regardless of the change in energetic intake. In addition, the relationships between the major fecal microbial groups and weight loss, change in fat mass, and change in lean mass were also evaluated. Weight loss was associated with a decrease in *Lactobacillus* group bacteria, whereas lean mass loss was associated with decreases in both bifidobacteria and *Lactobacillus* group bacteria. These findings confirm that the diet and energetic intake play an important role in modulation of the fecal microbiota. Finally, the potential of utilising the information on expression levels of selected stress genes in assessing the quality of probiotic products was evaluated. For this purpose, reverse transcription (RT)-qPCR methods were developed to study the expression of *clpL1* and *clpL2* stress genes in *Lactobacillus rhamnosus* VTT E-97800 cells after exposure to processing-related stress conditions or to freeze-drying. Heat treatments were performed with *L. rhamnosus* VTT E-97800 in laboratory scale, whereas acid treatments were performed both in laboratory and fermenter scale. RNA was extracted from fresh cells and freeze-dried powders. *clpL1* and *clpL2* transcripts were analysed by RT-qPCR using SYBR Green I. *clpL1* was induced in *L. rhamnosus* VTT E-97800 cells exposed to 50°C and to a much lesser extent in cells exposed to 47°C. No induction was observed for *clpL2* during either acid or heat treatment in any of the conditions applied. RNA isolation from freeze-dried powders was unsuccessful, although several attempts were made with high quality products. These results suggest that developing quality indicators for probiotic products based on differences in the expression of stress genes will be a challenging task, since rather harsh conditions are apparently needed to detect differences in the gene expression. In addition, the unsuccessful RNA isolation from freeze-dried powders hampers the applicability of this technique in the quality control of probiotic products.
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PUBLICATION I

The currently used commercial DNA-extraction methods give different results of clostridial and actinobacterial populations derived from human fecal samples

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RESEARCH ARTICLE

The currently used commercial DNA-extraction methods give different results of clostridial and actinobacterial populations derived from human fecal samples

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gut microbiota; clostridia; *Actinobacteria*; DNA-extraction; storage.

Abstract

Recently several human health-related microbiota studies have had partly contradictory results. As some differences may be explained by methodologies applied, we evaluated how different storage conditions and commonly used DNA-extraction kits affect bacterial composition, diversity, and numbers of human fecal microbiota. According to our results, the DNA-extraction did not affect the diversity, composition, or quantity of *Bacteroides* spp., whereas after a week's storage at −20 °C, the numbers of *Bacteroides* spp. were 1.6–2.5 log units lower ($P < 0.05$). Furthermore, the numbers of predominant bacteria, *Eubacterium rectale* (Erec)-group, *Clostridium leptum* group, bifidobacteria, and *Atopobium* group were 0.5–4 log units higher ($P < 0.05$) after mechanical DNA-extraction as detected with qPCR, regardless of storage. Furthermore, the bacterial composition of Erec-group differed significantly after different DNA-extractions; after enzymatic DNA-extraction, the most prevalent genera detected were *Roseburia* (39% of clones) and *Coprococcus* (10%), whereas after mechanical DNA-extraction, the most prevalent genera were *Blautia* (30%), *Coprococcus* (13%), and *Dorea* (10%). According to our results, rigorous mechanical lysis enables detection of higher bacterial numbers and diversity from human fecal samples. As it was shown that the results of clostridial and actinobacterial populations are highly dependent on the DNA-extraction methods applied, the use of different DNA-extraction protocols may explain the contradictory results previously obtained.

Introduction

In the past 10 years, there has been a wealth of studies in which the relationship between the human gut microbiota and human health has been investigated. After the findings of Turnbaugh *et al.* (2006) and Ley *et al.* (2006) that the relative proportion of *Bacteroidetes* decreased and the relative proportion of *Firmicutes* increased in obese mice (Ley *et al.*, 2005; Turnbaugh *et al.*, 2006) and men (Ley *et al.*, 2006) as compared to their lean counterparts, obesity-related gut microbiota studies have drawn a lot of attention. After the initial findings, there have been several related studies in which the findings have been similar (Armougom *et al.*, 2009; Santacruz *et al.*, 2009; Turnbaugh *et al.*, 2009; Balamurugan *et al.*, 2010; Santacruz *et al.*, 2010) to those of Ley *et al.* (2005, 2006) and Turnbaugh *et al.* (2006). Moreover, there have also been studies in which the findings have been contradictory or there has not been any statistically significant differences between *Firmicutes* and *Bacteroidetes* in obese and normal weight people (Duncan & Flint, 2008; Duncan *et al.*, 2008; Zhang *et al.*, 2009; Santacruz *et al.*, 2010; Schwiertz *et al.*, 2010). Some differences may be explained by the different detection methods applied [i.e. clone libraries (Ley *et al.*, 2005, 2006; Turnbaugh *et al.*, 2009; Zhang *et al.*, 2009) vs. quantitative PCR (Armougom *et al.*, 2009; Santacruz *et al.*, 2009; Zhang *et al.*, 2009; Balamurugan *et al.*, 2010; Santacruz *et al.*, 2010; Schwiertz *et al.*, 2010) vs. FISH (Duncan *et al.*, 2008)] and

different targets (Phylum *Bacteroidetes* vs. genus *Bacteroides* and Phylum *Firmicutes* vs. Families *Ruminococcaceae* and *Lachnospiraceae* + Incertae Sedis XIV (Clostridial clusters IV and XIV, respectively (Collins *et al.*, 1994) in addition to lactobacilli) and by different study populations, because for example in Europe differences in microbiota can be seen in people living in different areas (Mueller *et al.*, 2006).

As *Bacteroidetes* as Gram-negative and *Firmicutes* as Gram-positive bacteria have different cell wall structures and compositions, the optimal DNA-extraction method for the two groups is different. Gram-negative bacteria are more easily lyzed and if too rigorous DNA-extraction method is used, it may result in detecting lower numbers and diversity of *Bacteroidetes* species. With *Firmicutes*, instead, more rigorous DNA-extraction methods are needed, especially when the matrix is as complex as a human fecal sample. The most commonly used commercial fecal DNA-extraction kit (QIAamp DNA Stool Mini kit; Qiagen, Hilden, Germany, based on heat lysis and enzymatic digestion), which is nowadays considered as the 'golden standard' (Dridi *et al.*, 2009), has been originally validated using universal-denaturing gradient gel electrophoresis (DGGEs; Li *et al.*, 2003), and/or spiking experiments (McOrist *et al.*, 2002). However, more recent studies have been shown that mechanical cell disruption results in the detection of the highest bacterial diversity. Furthermore, as compared to enzymatic DNA-extraction, significantly improved DNA-extraction efficiency of *Clostridium leptum* group (Salonen *et al.*, 2010) [clostridial cluster IV (Collins *et al.*, 1994)] and methanogens (Dridi *et al.*, 2009) is obtained.

The effect of various storage temperatures on the fecal microbiota quantity and composition has also been studied. However, the results are partly contradictory. In a few recent studies, it was shown that storage for a short period of time at different temperatures does not significantly influence the bacterial community structure as detected with pyrosequencing (Roesch *et al.*, 2009; Lauber *et al.*, 2010). In contrast, in several older studies, the storage temperature has been shown to influence the results derived from gut microbiota as detected with terminal-restriction fragment length polymorphism, single-stranded conformation polymorphism analysis, and real-time PCR (Ott *et al.*, 2004; Molbak *et al.*, 2006; Roesch *et al.*, 2009).

The aim of this study was to evaluate whether different storage conditions and the DNA-extraction methods affect the detection of *Firmicutes* [*Eubacterium rectale* – *Blautia coccoides* group (*Lachnospiraceae*), *C. leptum* group (*Ruminococcaceae*), and lactobacilli], *Bacteroides,* bifidobacteria, and *Atopobium* group in human fecal samples. Furthermore, two new PCR-DGGE methods – for *Bacteroides* spp. and *C. leptum* –group – were optimized and validated in this study.

Materials and methods

Bacterial strains

The 83 bacterial reference strains used for the optimization and validation of PCR and PCR-DGGE for *Bacteroides* spp. and *C. leptum* group and quantitative PCR (qPCR) of predominant bacteria, *Bacteroides* spp., *C. leptum* group, and *E. rectale* – *B. coccoides* group, bifidobacteria and *Atopobium* group are listed in Supporting Information, Table S1. After optimization, negative and positive controls were included in the experiments with samples as well.

Human fecal samples

The fecal samples for sample storage and DNA-extraction analyses were obtained fresh from a healthy female subject (subject 1) (44 years old) and a healthy male subject (subject 2) (51 years old). The main recruiting criterion was a good (normal) intestinal balance (absence of repeating and/or persisting gastrointestinal symptoms). The exclusion criteria were regular GI-tract symptoms, lactose-intolerance, celiac disease, and antimicrobial therapy during the last 2 months prior to the sampling point. The subjects defecated into a plastic container, which was made anaerobic with gas-generators (Anaerocult A mini, Merck, Darmstadt, Germany) placed on the lid of the container. The samples were transported to the laboratory, homogenized, and divided into subsamples in an anaerobic workstation (Don Whitley Scientific Ltd, Shipley, UK) within 0–4 h from the defecation. Part of the sample was further processed fresh, second subsample was stored at 4 °C for 2 days after which it was transferred to −70 °C, third subsample was stored at −20 °C for a week and thereafter at −70 °C, and fourth subsample was transferred directly to −70 °C for storage. The study plan is presented in Fig. 1.

The fecal samples for optimization and validation of *Bacteroides* spp. and *C. leptum* (Clept) group protocols were obtained from two healthy females (34 and 39 years old). The larger study group for diversity and stability studies of *Bacteroides* spp. and Clept-group consisted of 10 subjects that were 34–62 years of age (three males and seven females). Fecal samples were obtained on three occasions 3 months apart (0, 3, and 6 months). The main recruiting and exclusion criteria were as above. The samples were collected as earlier and maintained at −70 °C until analyzed. Human studies were approved by the ethical committee of VTT Technical Research Centre of Finland, Espoo, Finland.

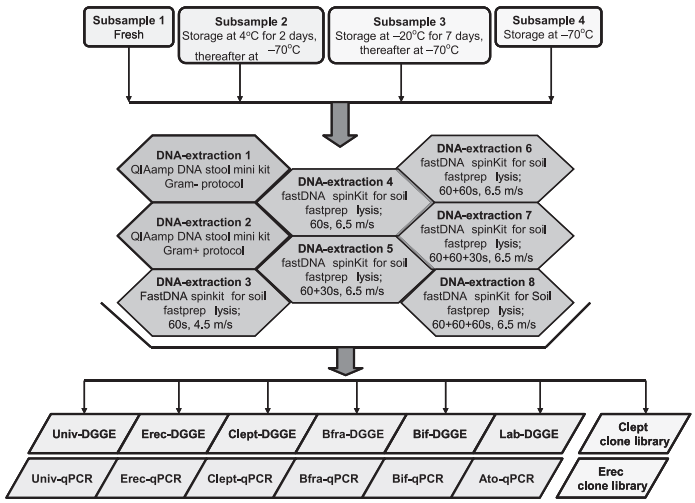

Fig. 1. The study plan. Univ, predominant bacteria; Erec, *Eubacterium rectale – Blautia coccoides* group; Clept, *Clostridium leptum* group; Bfra, *Bacteroides* spp. ; Lab, *Lactobacillus* group (comprises of genera *Lactobacillus, Leuconostoc, Pediococcus*, and *Weissella*); Ato, *Atopobium* group (comprises e.g. of genera *Atopobium*, *Eggerthella*, and *Collinsella*).

DNA-extraction

For DNA-extraction experiments six different protocols (FastPrep lysis) (1) 60 s 4.5 m s^{-1}; (2) 60 s 6.5 m s^{-1}; (3) 60 s + 30 s 6.5 m s^{-1}; (4) 60 s + 60 s 6.5 m s^{-1}; (5) 60 s + 60 s + 30 s 6.5 m s^{-1}; (6) 60 s + 60 s + 60 s 6.5 m s^{-1}) with FastDNA Spin kit for Soil (MP Biomedicals, Solon, OH; from hereon referred to as 'mechanical DNA-extraction') and two different protocols (1) Gram-negative bacteria and (2) Gram-positive bacteria with QIAamp DNA Stool Mini kit (Qiagen, from hereon referred to as 'enzymatic DNA-extraction') were evaluated from different storage conditions (Fig. 1). All the DNA-extraction experiments were performed in duplicate. DNA was extracted as previously described (Maukonen *et al*., 2006b) from the 0.2 g of samples that were used for studying of the diversity and stability of the Clept-group and *Bacteroides* spp.

PCR of *C. leptum* group (Clept-group)

Six different primer combinations, four $MgCl_2$-concentrations, 11 different annealing temperatures, and three different cycle numbers were tested in preliminary PCR-DGGE experiments. Partial 16S rRNA gene of Clept-group was PCR-amplified for DGGE using primer pairs Clept-933 f and Clept-1240-r+GC (Table S2) in addition to Clept-933 f+GC and Clept-1240-r. Optimized PCR amplifications were performed in a total volume of 30 μL containing 1 μL of appropriately diluted template DNA, 0.4 μM of both primers, 0.2 mM dNTP, 1.25 units of *Taq* polymerase (Invitrogen, Carlsbad, CA) in a reaction buffer with 20 mM Tris–HCl (pH 8.4), 50 mM KCl, and 2.5 mM $MgCl_2$. The PCR program consisted of initial denaturing at 94 °C for 5 min, followed by 30 cycles of denaturing at 94 °C for 45 s, primer annealing at 60 °C for 30 s and elongation at 72 °C for 60 s, and a final extension for 30 min at 72 °C.

PCR of *Bacteroides* spp.

Ten different primer combinations, six $MgCl_2$-concentrations, 12 different annealing temperatures, and two different cycle numbers were tested in preliminary PCR-DGGE experiments. Partial 16S rRNA gene of *Bacteroides* spp. was PCR-amplified for DGGE using primer pairs Bact596f and Bacto1080r+GC in addition to Bact596f+GC and Bacto1080r (Table S2). Optimized PCR amplifications were performed in a total volume of 30 μL containing 1 μL of appropriately diluted template DNA, 0.4 μM of both primers, 0.2 mM dNTP, 1.25 units of *Taq* polymerase (Invitrogen) in a reaction buffer with 20 mM Tris –HCl (pH 8.4), 50 mM KCl, and 1.5 mM $MgCl_2$. The PCR program consisted of initial denaturing at 94 °C for 5 min, followed by 30 cycles of denaturing at 94 °C for 45 s, primer annealing at 58 °C for 30 s and elongation at 72 °C for 60 s, and a final extension for 30 min at 72 °C.

PCR of predominant microbiota, *E. rectale* – *B. coccoides* clostridial group (Erec-group), bifidobacteria, and *Lactobacillus* group

Partial 16S rRNA gene for the analysis of predominant bacteria was amplified using primers U968-f+GC and U1401-r (Table S2) as described previously (Mättö *et al.*, 2005) and primers 358f+GC and 534r (Table S2) as previously described (Maukonen *et al.,* 2006a) Erec-group was amplified using primers Ccoc-f and Ccoc-r+GC, bifidobacteria with primers Bif164-f and Bif662-GC-r, and *Lactobacillus* group with primers Lac1 and Lac2GC (Table S1) as previously described (Maukonen *et al.,* 2006b; Maukonen *et al.*, 2008),

Cloning of the PCR-amplified products

PCR amplicons of Clept-group and *Bacteroides* spp. for DGGE-method validation and PCR amplicons of Clept-group and Erec-group for DNA-extraction method validation were purified using a Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Thereafter the cloning and analysis of the clones were performed as previously described (Maukonen *et al.,* 2006b). Altogether 144 clones were collected from each specific PCR and sequenced. The good quality sequences (*c.* 110–140 clones / PCR) were subjected to ClustalW analysis (http://www.ebi.ac.uk/Tools/clustalw2/index.html?) for checking of the sequence similarities. All unequal sequences were thereafter identified through the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the blast algorithm (Altschul *et al.*, 1990) or using the 'Classifier' tool of the Ribosomal Database Project (RDP) II (Wang *et al.*, 2007). Library compare of RDPII (Cole *et al.*, 2009) was used for the classification of sequences derived from DNA-extraction optimization into the phylogenetically consistent higher-order bacterial taxonomy. Each different clone was deposited in the GenBank database and the sequences are available under the accession numbers JN206701–JN207127. Phylogenetic analyses were performed using the Kodon software (Applied Mathematics, Sint-Martens-Latem, Belgium).

DGGE analysis of 16S rRNA gene fragments

DGGE analyses of predominant bacteria, Erec-group, *Lactobacillus* group, and bifidobacteria were performed as described previously (Maukonen *et al.*, 2008). The primer pair Clept-933 f and Clept-1240-r+GC was chosen for the Clept-group DGGE analysis and primer pair Bact596f and Bacto1080r+GC for the DGGE analysis of *Bacteroides* spp. because of specificity and optimal migration. DGGE analyses of Clept-group and *Bacteroides* spp. were performed as previously described (Maukonen *et al.*, 2008), with several modifications. Various denaturing gradients were tested and subsequently denaturing gradient from 30% to 60% [where 100% is 7 M urea and 40% (v/v) deionized formamide] was chosen because of optimal amplicon migration and differentiation. Similarity of the PCR-DGGE profiles of the samples obtained from a single subject at different sampling points was compared to evaluate the diversity and temporal stability of the selected bacterial populations. The comparison of the profiles was performed by calculating a similarity percentage using BioNumerics software version 5.1 (Applied Mathematics BVBA). Clustering was performed with Pearson correlation and the unweighted-pair group method (UPGMA). Amplicons with the total surface area of at least 1% were included in the similarity analysis.

After sequence analysis of the clones from the newly developed Clep-DGGE and *Bacteroides* DGGE, all the clones with different sequences were subjected to either *Bacteroides* DGGE or Clept-DGGE, after which the migration of each clone was compared to the migration of different amplicons in the original sample.

Quantitative PCR (qPCR) of 'all' bacteria, Clept-group, Erec-group, *Bacteroides* spp., bifidobacteria, and *Atopobium* group

The specificity of various different primer pair combinations for quantitative amplification were optimized and validated for amplification of partial 16S rRNA gene of predominant bacteria, Clept-group, Erec-group, *Bacteroides* spp., bifidobacteria, and *Atopobium* group using the bacteria listed in Table S1. Subsequently the following primer pairs were chosen; 358f and 534r for predominant bacteria, Clept-F and Clept-R3 for Clept-group, g-Ccoc-F and g-Ccoc-R for Erec-group, g-Bfra-F and g-Bfra-R for *Bacteroides* spp., Bifid-f and Bifid-r for bifidobacteria, and Atopo-f and Atopo-r for *Atopobium* group (Table S2). High Resolution Melting Master kit (Roche, Mannheim, Germany) using Sybr-Green like chemistry with Reso-Light high-resolution melting dye with $MgCl_2$ concentration of 2.5 mM (predominant bacteria and Clept-group), 1.9 mM (Erec-group), 3.1 mM (*Bacteroides* spp.), 2.5 mM (bifidobacteria), or 2.5 mM (*Atopobium* group) were used according to the manufacturer's instructions in LightCycler 480 II instrument (Roche). The initial denaturing at 95 °C for 10 min, high-resolution melting (95 °C 1 min, 40 °C 1 min, 65 °C 1 s, 95 °C), and cooling (40 °C, 30 s) steps were identical to all the protocols. The rest of the amplification protocols were as follows: predominant bacteria: initial denaturing at 45 cycles of denaturing at 95 °C for 15 s, primer annealing at 50 °C for 20 s and elongation at 72 °C for 25 s; Clept-group: 45 cycles of

denaturing at 95 °C for 10 s, primer annealing at 60 °C for 15 s and elongation at 72 °C for 20 s; Erec-group: 45 cycles of denaturing at 95 °C for 15 s, primer annealing at 64 °C for 20 s and elongation at 72 °C for 25 s; *Bacteroides* spp.: 45 cycles of denaturing at 95 °C for 15 s, primer annealing at 58 °C for 20 s and elongation at 72 °C for 25 s; bifidobacteria: 45 cycles of denaturing at 95 °C for 10 s, primer annealing at 60 °C for 15 s and elongation at 72 °C for 20 s; and *Atopobium* group: 45 cycles of denaturing at 95 °C for 10 s, primer annealing at 64 °C for 15 s and elongation at 72 °C for 20 s. All the qPCRs were performed in duplicate (from duplicate DNA-extractions that is all together four replicates). Negative and positive controls were included in all the experiments. Standard curves were created with the help of DNA extracted from a known number of culturable representatives of the bacterial groups (*B. coccoides* for predominant bacteria and Erec-group, *Anaerotruncus colihominis* for Clept-group, *Bacteroides fragilis* for *Bacteroides* spp., *Bifidobacterium longum* for bifidobacteria and *Atopobium parvulum* for *Atopobium* group) and thereafter qPCR was performed in duplicate from serially diluted standard-DNA as described earlier.

Statistical analysis

The qPCR data were transformed to logarithmic scale to be able to use parametric statistical methods. Mean and standard deviation were calculated for each experiment. Student's *t*-test (two-sample assuming equal or unequal variances, depending on data-set) was used for the statistical analyses of the results.

Results

Optimization and validation of Clept-group and *Bacteroides* spp. specific DGGEs

When the specificity of primer pairs Clept-933 f–Clept-1240-r+GC and Bact596f–Bacto-1080r+GC was evaluated using reference strains, the primers gave positive PCR results for all the target bacteria belonging to the Clept-group or genus *Bacteroides*, respectively. No false-positive results were obtained. All amplicons of the reference strains for Clept-group and *Bacteroides* spp. migrated differently and could thus be distinguished (data not shown). Two samples were cloned after Clept PCR and *Bacteroides* PCR to validate the specificity. All the sequenced 204 Clept PCR clones belonged to the *C. leptum* clostridial cluster IV and 240 *Bacteroides* PCR clones to *Bacteroides* spp. The DGGE profiles of the cloned samples and the sequence information obtained from the cloning of Clept-group are presented in Figs S1 and S2. The same information in regard of *Bacteroides* spp. is presented in Figs S3 and S4.

Clept-group and *Bacteroides* spp. diversities were on average 20.9 and 9.2 amplicons per sample, respectively. Intraindividual similarity of the follow-up samples was higher than interindividual similarity in both groups that is in 8/10 subjects all the samples from a given subject clustered together (each individual created his/her own cluster, data not shown). The intraindividual similarities of Clept-group and *Bacteroides* spp. of samples taken 3 months apart were $87.5 \pm 9.2\%$ and $85.4 \pm 12.1\%$, respectively, whereas the intraindividual similarities of Clept-group and *Bacteroides* spp. of samples taken half a year apart were in average $81.7 \pm 11.7\%$ and $82.0 \pm 16.2\%$, respectively.

The effect of different DNA-extraction methods and storage conditions on DNA yield

The use of different mechanical lysis methods (DNA-extraction protocols 2–8; Fig. 1) did not affect the DNA yield from fresh samples, whereas with stored samples both the kit and different modifications to the kits caused variations on the DNA yield. The highest DNA yield was achieved with the most rigorous mechanical lysis (DNA-extraction protocol 8; 3×60 s; 6.5 m s^{-1}) at all storage temperatures. The DNA yield was about 60% after gentle mechanical lysis (60 s 4.5 m s^{-1}; $P < 0.05$) and 20% after enzymatic lysis ($P < 0.05$) as compared to the rigorous DNA-extraction. The storage temperature did not cause significant differences in the DNA yield when the same protocol was applied to the same sample stored at different temperatures.

The effect of different DNA-extraction methods and storage conditions on the diversity and quantity of predominant bacteria

The storage conditions had a significant effect on the diversity of the predominant fecal bacteria of the studied subjects. With mechanical DNA-extraction, the predominant bacterial diversity of fresh samples and samples stored at −70 °C was significantly higher ($P < 0.05$) than those stored initially at −20 or at 4 °C. When enzymatic DNA-extraction was applied, significant, storage temperature dependent, differences ($P < 0.05$) were seen only with one subject. However, the predominant bacterial diversity of both subjects was significantly higher ($P < 0.005$) after mechanical DNA-extraction than after enzymatic DNA-extraction at all storage temperatures tested. Furthermore, the bifidobacteria-associated bands in the predominant bacterial DGGE-profile were weaker when a gentle mechanical (60 s lysis) DNA-extraction

protocol was used as compared to the more rigorous mechanical lysis (60 s + 60 s + 60 s, data not shown). After clustering of the predominant bacterial DGGE profiles of both subjects after all different storage – DNA-extraction – PCR combinations, the samples clustered primarily according to the PCR primers used (i.e. V_6–V_8 region vs. V_3–V_5 region), secondarily according to the DNA-extraction kit used (regardless of modifications) and thirdly according to the individual, and storage conditions. The similarity between the same samples obtained after DNA-extraction with different commercial kits (e.g. person A, mechanical lysis vs. enzymatic lysis) was 32–38% after amplification of V_6–V_8 region (Fig. 2) and 48–54% after PCR of V_3–V_5 region (Fig. 2), whereas the similarity of the same samples between different storage conditions was 89–98% when the similar DNA-extraction protocol was applied (data not shown). Furthermore, the storage conditions did not have a significant effect on the quantity of predominant bacteria as detected with a predominant bacterial qPCR, but the difference between quantity of predominant bacteria after DNA-extraction using different DNA-extraction kits was significant at all storage temperatures ($P < 0.005$; Fig. 3; results after storage at −70 °C are presented).

The effect of different DNA-extraction methods and storage conditions on the diversity, composition, and quantity of Erec-group bacteria

After clustering of the Erec-group DGGE profiles of both subjects after all different storage – DNA-extraction combinations, the samples clustered in three major cluster: cluster 1: subject 1, enzymatic DNA-extraction; cluster 2: subject 2, enzymatic DNA-extraction; and cluster 3: subjects 1 and 2, mechanical DNA-extraction (Fig.

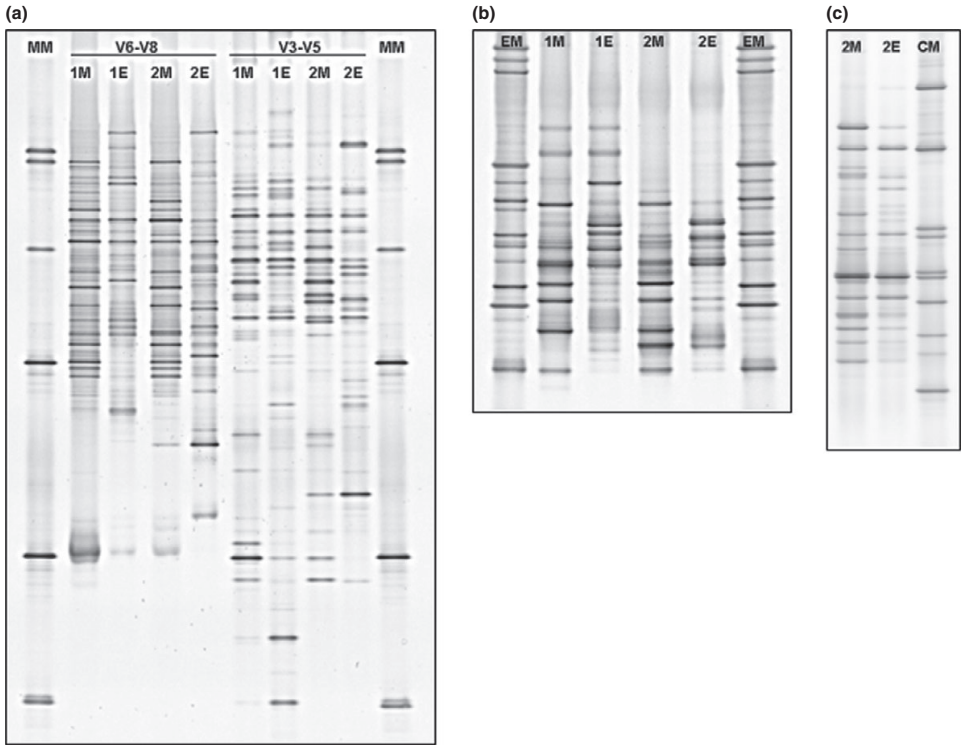

Fig. 2. (a) Predominant bacterial DGGE profiles as detected with amplification of 16S rRNA gene variable region V_6–V_8 (lanes 1–4) or V_3–V_5 (lanes 5–8); (b) *Eubacterium rectale* – *Blautia coccoides* group-specific DGGE profiles; and (c) *Clostridium leptum* group-specific DGGE profiles. 1M, sample from subject 1 after mechanical DNA-extraction; 1E, sample from subject 1 after enzymatic DNA-extraction; 2M, sample from subject 2 after mechanical DNA-extraction; 2E, sample from subject 1 after enzymatic DNA-extraction; MM, marker, EM, Erec-group marker, CM, Clept-group marker; all these samples were stored at −70 °C.

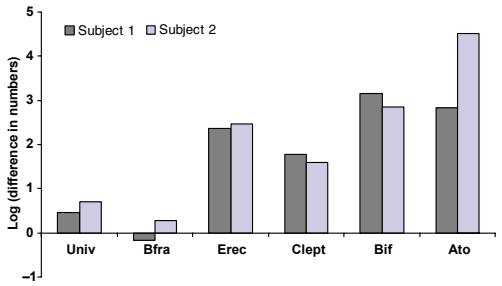

Fig. 3. Difference between numbers of bacterial groups obtained after different DNA-extraction protocols and qPCR from samples stored at −70 °C. The results are expressed as log-values (mechanical lysis – enzymatic lysis; i.e. positive number indicates more efficient mechanical lysis). All other differences, except *Bacteroides* spp., were statistically significant ($P < 0.05$). Univ, predominant bacteria, Bfra, *Bacteroides* spp., Erec, *Eubacterium rectale – Blautia coccoides* group, Clept, *Clostridium leptum* group, Bif, bifidobacteria, Ato, *Atopobium* group.

S5). Within cluster 3, there were further two sub-clusters for differentiating the two individuals. The similarity of the same samples obtained after DNA-extraction with different kits (within the same storage conditions) was 42–53% (Fig. 2), whereas the similarity of the same sample after different storage conditions was > 95% when similar DNA-extraction protocol was applied (Fig. S5). Clone library analysis confirmed the different bacterial compositions detected with DGGE (Table 1). After enzymatic DNA-extraction, the most prevalent genera detected were *Roseburia* (39% of clones) and *Coprococcus* (10%), whereas 37% of the clones belonged to unclassified *Lachnospiraceae*. After mechanical DNA-extraction, the most prevalent bacterial genera of Erec-group were *Blautia* (30% of clones), *Coprococcus* (13%), and *Dorea* (10%), whereas 27% of the clones belonged to unclassified *Clostridiales*; only 10% of the clones belonged to genus *Roseburia* and 11% to unclassified *Lachnospiraceae*. A few clones (1.6%) belonging to the genus *Anaerostipes* were detected only after enzymatic DNA-extraction. The Erec-group diversity stayed about the same regardless of the DNA-extraction protocol applied, as detected with both DGGE and clone libraries (Fig. S6). The storage conditions did not have a significant effect on the quantity of Erec-group bacteria as detected with qPCR, when similar protocols were compared. However, the numbers of Erec-group bacteria were significantly higher after mechanical DNA-extraction ($P < 0.05$; ~2 log unit difference; Fig. 3) than after enzymatic DNA-extraction, regardless of the protocol and storage conditions applied.

Table 1. Phylogenetic classification as determined with the Library compare of RDPII (Cole *et al.*, 2009) of the *Eubacterium rectale – Blautia coccoides* group clone libraries derived from a single sample (library 1 = enzymatic DNA-extraction; library 2 = mechanical; and chemical DNA-extraction)

Rank	Name	Library 1	Library 2	Significance
Phylum	*'Firmicutes'*	126	126	1E0
Order	*Clostridiales*	126	126	1E0
Family	*'Lachnospiraceae'*	111	54	6E−14
Genus	*Dorea*	1	12	1.83E−3
Genus	*Coprococcus*	13	16	5.55E−1
Genus	*Anaerostipes*	2	0	2.5E−1
Genus	*Roseburia*	49	12	6.68E−8
	Unclassified *'Lachnospiraceae'*	46	14	NA
Family	*Incertae Sedis XIV*	3	38	5.63E−9
Genus	*Blautia*	3	38	5.63E−9
	Unclassified *Clostridiales*	12	34	NA

The effect of different DNA-extraction methods and storage conditions on the diversity, composition, and quantity of Clept-group bacteria

After clustering of the Clept-group DGGE profiles of both subjects after all different storage – DNA-extraction combinations, the samples clustered primarily according to the individual, secondarily (within the primary clusters) according to the used DNA-extraction kit and thirdly according to the storage conditions. The similarity of the same samples obtained after DNA-extraction with different kits (at the same storage condition) was 80–87% (Fig. 2), whereas the similarity of the same sample after different storage conditions was > 94% when similar DNA-extraction protocol was applied (Fig. S7), except for sub-samples stored initially at 4 °C for 2 days. The similarity of samples stored at 4 °C was 84–93% as compared to the same samples stored at −20 or at −70 °C (using identical DNA-extraction protocol). Clone library analysis confirmed the partly different bacterial composition detected with DGGE (Table 2). The most prevalent Clept-group genera detected after mechanical DNA-extraction were *Faecalibacterium* (39%) and *Subdoligranulum* (37%), whereas after enzymatic DNA-extraction 80% of the clones grouped to genus *Subdoligranulum*. In addition, the diversity of the Clept-group was higher after mechanical DNA-extraction, as detected with both DGGE and clone libraries (Fig. S6). The storage conditions did not have a significant effect on the quantity of Clept-group bacteria as detected with qPCR, when similar protocols were compared. However, the numbers of Clept-group bacteria were significantly higher after

Table 2. Phylogenetic classification as determined with the Library compare of RDPII (Cole *et al.*, 2009) of the *Clostiriudm leptum* group clone libraries derived from a single sample (library 1 = enzymatic DNA-extraction; library 2 = mechanical and chemical DNA-extraction)

Rank	Name	Library 1	Library 2	Significance
Phylum	'*Firmicutes*'	112	116	9.99E−1
Class	'*Clostridia*'	111	113	8.96E−1
Order	*Clostridiales*	110	113	9.5E−1
Family	'*Ruminococcaceae*'	110	112	8.98E−1
Genus	*Faecalibacterium*	6	45	1.98E−9
Genus	*Subdoligranulum*	90	43	4.13E−11
Genus	*Butyricicoccus*	1	2	6.52E−1
Genus	*Anaerotruncus*	1	0	4.83E−1
	Unclassified '*Ruminococcaceae*'	12	22	NA
	Unclassified *Clostridiales*	0	1	NA
	Unclassified '*Clostridia*'	1	0	NA
	Unclassified '*Firmicutes*'	1	3	NA

mechanical DNA-extraction ($P < 0.05$; ~1.5 log unit difference; Fig. 3) than after enzymatic DNA-extraction, regardless of the protocol and storage conditions applied.

The effect of different DNA-extraction methods and storage conditions on the diversity and quantity of *Bacteroides* spp.

The storage conditions and different DNA-extraction methods did not have an effect on the diversity and composition of *Bacteroides* spp. All the profiles of a given person, regardless of the protocol or storage conditions applied, were similar (similarity with Pearson correlation > 90%). However, the storage conditions greatly affected the numbers of the *Bacteroides* spp. The highest numbers of *Bacteroides* spp. were obtained from fresh samples as detected with qPCR, whereas after a week's storage at −20 °C, the numbers of *Bacteroides* spp. were significantly lower (1.6–2.5 log reduction, depending on the DNA-extraction modification) than those in fresh samples (Fig. S8).

The effect of different DNA-extraction methods and storage conditions on the diversity and/or quantity of bifidobacteria, *Lactobacillus* group, and *Atopobium* group

The different storage conditions and DNA-extraction methods did not affect the bifidobacterial diversity or the composition as detected with bifidobacteria-specific DGGE (data not shown). The storage conditions did not have a significant effect on the numbers of bifidobacteria when similar protocols were compared. However, the numbers of bifidobacteria were significantly higher ($P < 0.05$; ~3 log unit difference; Fig. 3) after mechanical DNA-extraction than after enzymatic DNA-extraction as detected with qPCR.

The diversity and composition of *Lactobacillus* group were not significantly affected, as detected with specific DGGE, by the storage conditions when mechanical DNA-extraction was applied. After enzymatic DNA-extraction, the samples did not amplify with the *Lactobacillus* group-specific PCR at all.

The numbers of *Atopobium* group bacteria were significantly affected by both storage conditions and used DNA-extraction protocols as detected with qPCR. After mechanical DNA-extraction, the number of *Atopobium* group bacteria was significantly higher ($P < 0.05$; 2.5–4.5 log unit difference; Fig. 3) at all evaluated storage conditions. Furthermore, the numbers of *Atopobium* group bacteria were significantly higher ($P < 0.05$; > 1 log unit difference) after initial storage at −20 °C for a week or at 4 °C for 2 days than those from fresh samples or samples stored solely at −70 °C.

Discussion

Recently there have been several human health-related microbiota studies with partly contradictory results regarding obesity-related microbiota and bifidobacterial abundance of baby microbiota. As it is likely that at least some of the differences could be explained by the methodology applied, we evaluated the impact of commonly used commercial DNA-extraction kits (with several modifications) and storage temperatures on most prevalent human gut microbial groups. There are a few recent (Dridi *et al.*, 2009; Ariefdjohan *et al.*, 2010; Salonen *et al.*, 2010) and older (Zoetendal *et al.*, 2001; McOrist *et al.*, 2002; Li *et al.*, 2003) studies in which the effect of DNA-extraction on results derived from human fecal samples has been studied. However, in those studies where the commonly used commercial kits have been applied, only the 'universal' level of predominant bacteria has been studied (McOrist *et al.*, 2002; Li *et al.*, 2003; Ariefdjohan *et al.*, 2010), or the study has focused on a specific microbial group, such as methanogens (Dridi *et al.*, 2009). On the other hand, in a recent study (Salonen *et al.*, 2010) in which the gut microbiota was studied in more detail, different DNA-extraction protocols – as compared to the ones we used – were applied. The impact of storage conditions on diversity and composition of fecal microbiota has also been studied (Molbak *et al.*, 2006; Roesch *et al.*, 2009; Lauber *et al.*, 2010), but to our knowledge, this is the first study in which both the effect of DNA-extraction and storage conditions on diversity, composition, and numbers of the most pre-

valent fecal bacterial groups are studied from the same samples.

Our findings showed that the commercial DNA-extraction kits had a significant effect on both composition and numbers of abundant human fecal microbial groups. The numbers and composition of *Bacteroides* spp. were not significantly affected by the various DNA-extraction protocols, which is in line with the previous findings (Salonen *et al.*, 2010). However, the storage temperature had a significant effect on the quantity of *Bacteroides* spp., causing > 1 log reduction with all tested storage condition as compared to the same sample as fresh. These findings may partly explain why no *Bacteroidetes* were found in the study of Gill *et al.* (2006). The clostridial populations, namely *E. rectale – B. coccoides* (Erec) group and *C. leptum* (Clept) group, were significantly affected in both composition and numbers by the DNA-extraction protocol used. The numbers of both clostridial groups were significantly lower when enzymatic DNA-extraction kit was used (~2 log reduction) as compared to mechanical DNA-extraction kit. In addition, the composition of Erec-group was different when different commercial kits were applied to the same samples. The most prevalent genus detected after enzymatic DNA-extraction was *Roseburia* (39% of clones), whereas after mechanical DNA-extraction, the most prevalent genus was *Blautia* (30% of clones). The same phenomenon was also noticed with the Clept-group bacteria; the most prevalent Clept-group genera detected after mechanical DNA-extraction were *Faecalibacterium* (39%) and *Subdoligranulum* (37%), whereas after enzymatic DNA-extraction, 80% of the clones grouped to genus *Subdoligranulum.* The higher proportion of genus *Faecalibacterium* after rigorous DNA-extraction has also been noted in earlier studies with different protocols (Salonen *et al.*, 2010).

Most of the obesity-related studies that have obtained similar results, that is, that the relative proportion of Bacteroidetes decreases and the relative proportion of Firmicutes increases in obese human (Ley *et al.*, 2006), have used QIAmp DNA Stool Mini Kit (Santacruz *et al.*, 2009; Zhang *et al.*, 2009; Balamurugan *et al.*, 2010; Santacruz *et al.*, 2010), whereas in those studies, in which there has been no difference between obese and lean subjects or the results have been contradictory to the initial findings, a more rigorous DNA-extraction protocol has been applied (Mai *et al.*, 2009; Schwiertz *et al.*, 2010; Arumugam *et al.*, 2011) or the samples have been studied with fluorescent *in situ* hybridization (Duncan *et al.*, 2007; Collado *et al.*, 2008; Duncan *et al.*, 2008; Mai *et al.*, 2009). As our results demonstrate that the DNA-extraction protocol has a major effect on the clostridial populations while having no effect on the *Bacteroides* population, it may be that the contradictory results are, at least partly, caused by different DNA-extraction protocols.

Another recent example of contradictory findings has involved the bifidobacterial populations of baby feces. Bifidobacterial populations have been found to constitute a dominant part of baby feces many decades ago by culture-based methods (Bullen *et al.*, 1976; Stark & Lee, 1982). With molecular techniques the results have, however, been partly contradictory. There are numerous studies conducted with molecular techniques in which bifidobacteria have been shown to dominate baby fecal microbiota (Harmsen *et al.*, 2000b; Favier *et al.*, 2002; Magne *et al.*, 2006; Fallani *et al.*, 2010), but then there are those in which bifidobacteria have been found to constitute only a minor part of the infant microbiota (Palmer *et al.*, 2007). Similarly to the obesity issue, also this inconsistency may result from different DNA-extraction techniques applied. In those molecular studies where bifidobacteria have been shown to predominate in the baby feces, mechanical DNA-extraction has been applied (Favier *et al.*, 2002; Magne *et al.*, 2006) or the samples have been studied with FISH (Harmsen *et al.*, 2000b; Fallani *et al.*, 2010). In those studies where the authors have concluded that bifidobacteria constitute only a minor part of the baby fecal microbiota, enzymatic DNA-extraction using the same commercial kit as in this study has been applied (Palmer *et al.*, 2007). Because our results showed that with enzymatic DNA-extraction, the number of bifidobacteria may be even 3 log units lower than with rigorous mechanical DNA-extraction, differences in DNA-extraction likely explain these contradictory results. In addition, Nakamura *et al.* (2009) showed that when enzymatic DNA-extraction was applied bifidobacterial abundance was 0.1–1.7%, whereas when FISH was applied to the same samples the bifidobacterial abundance increased to 20.7–83.5% in baby feces. Furthermore, previously it has been found (Salonen *et al.*, 2010) that with more rigorous mechanical disruption, the proportion of Actinobacteria (e.g., bifidobacteria and *Atopobium* group) increased. Interestingly, the protocol that yielded the lowest levels of Actinobacteria was based on the same kit that we used for enzymatic DNA-extraction (Salonen *et al.*, 2010). However, we used the kit according to the manufacturer's instructions, whereas Salonen *et al.* (2010) added 3 × 30 s bead beating and extended the duration of heat lysis in their study. Even with these modifications, the proportion of Actinobacteria was low.

The highest numbers of most of the studied bacterial groups were detected from fresh samples and from samples stored at −70 °C. Unexpectedly, the highest numbers of *Atopobium* group were detected after an initial storage at 4 °C for 2 days. The unexpected effect of storage conditions on *Atopobium* group numbers was confirmed with

FISH (data not shown). The numbers of *Atopobium* group bacteria were 5–6 log units higher in a sample that was initially stored at 4 °C for 2 days and thereafter at −70 °C and when DNA-extraction was performed mechanically (3 × 60 s) than on the same sample that was stored at −70 °C and the DNA-extraction was performed enzymatically. There was no clear explanation for this phenomenon in the literature. The only possibly relevant finding was that *Collinsella* spp. cell wall contains a unique A4β-type peptidoglycan (Kageyama *et al.*, 1999). Therefore it is possible that the cell wall structure is extremely difficult to lyse without the extra stress of storage at 4 °C. However, these results may explain, why in some studies the *Atopobium* group bacteria are not considered to be part of the normal dominant microbiota (Eckburg *et al.*, 2005), whereas in others, especially those conducted with FISH, *Atopobium* group bacteria are shown to constitute 1–8% of the total population of the human gut microbiota (Harmsen *et al.*, 2000a; Matsuki *et al.*, 2004; Lay *et al.*, 2005; Mueller *et al.*, 2006).

In conclusion, rigorous mechanical lysis enables detection of higher bacterial numbers and diversity from human fecal samples. As it was shown that the results of clostridial and actinobacterial populations are highly dependent on the DNA-extraction methods applied, the use of different DNA-extraction protocols may partly explain the contradictory results previously obtained in regard of obesity related and infant microbiota.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The cloned and sequenced bands from the *Clostridium leptum* group-specific PCR-DGGE of the subject B1 (n.d. = the clone is not visible in the community profile).

Fig. S2. The cloned and sequenced bands from the *Clostridium leptum* group-specific PCR-DGGE of the subject B2 (n.d. = the clone is not visible in the community profile).

Fig. S3. The cloned and sequenced bands from the genus *Bacteroides*-specific PCR-DGGE of the subject B1 (n.d. = the clone is not visible in the community profile).

Fig. S4. The cloned and sequenced bands from the genus *Bacteroides*-specific PCR-DGGE of the subject B2 (n.d. = the clone is not visible in the community profile).

Fig. S5. Clustering of Erec-group DGGE profiles of both subjects after different storage – DNA-extraction combinations.

Fig. S6. Rarefaction curves of different clones per sample.

Fig. S7. Clustering of Clept-group DGGE profiles of both subjects after different storage – DNA-extraction combinations.

Fig. S8. Difference in numbers of *Bacteroides* spp. after all different storage combinations and mechanical DNA-extraction.

Table S1. Bacterial pure cultures used in this study for optimization of group-specific PCR-DGGEs and real-time PCR methods.

Table S2. Primers used in the present study.

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PUBLICATION II

Habitual dietary intake is associated with the stool microbiota composition of monozygotc twins

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Habitual Dietary Intake Is Associated with Stool Microbiota Composition in Monozygotic Twins[1–4]

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Abstract

The impact of diet on the gut microbiota has usually been assessed by subjecting people to the same controlled diet and thereafter following the shifts in the microbiota. In the present study, we used habitual dietary intake, clinical data, qPCR, and denaturing gradient gel electrophoresis (DGGE) to characterize the stool microbiota of Finnish monozygotic twins. The effect of diet on the numbers of bacteria was described through a hierarchical linear mixed model that included the twin individuals, stratified by BMI, and their families as random effects. The abundance and diversity of the bacterial groups studied did not differ between normal-weight, overweight, and obese individuals with the techniques used. Intakes of energy, MUFA, (n-3) PUFA, (n-6) PUFA, and soluble fiber had significant associations with the stool bacterial numbers (e.g., increased energy intake was associated with reduced numbers of *Bacteroides* spp.). In addition, co-twins with identical energy intake had more similar numbers and DGGE-profile diversities of *Bacteroides* spp. than did the co-twins with different intake. Moreover, the co-twins who ingested the same amounts of SFA had very similar DGGE profiles of *Bacteroides* spp., whereas the co-twins with similar consumption of fiber had a very low bifidobacterial DGGE-profile similarity. In conclusion, our findings confirm that the diet plays an important role in the modulation of the stool microbiota, in particular *Bacteroides* spp. and bifidobacteria. J. Nutr. doi: 10.3945/jn.112.166322.

Introduction

The influence of the diet on the gastrointestinal tract microbiota composition, numbers, and activity has been discussed for several decades (1,2). Early culture-based studies comparing defined diets (e.g., Japanese versus Western) did not show major differences in the composition of the resident stool microbiota (3), whereas chemically defined diets produced diminished stool mass associated with compositional changes in the microbiota (4). Advances in molecular microbiologic techniques have expanded our knowledge on the gut microbial ecology (5), thus starting a new era of the study of the impact of diet and dietary changes on the resident microbiota. At the same time, the diet itself has been changing worldwide as a result of alterations in lifestyle, agricultural practices, and population growth (6). Controlled diets, such as those having high protein and reduced carbohydrate content (7), or diets differing in nondigestible carbohydrate content (8) have been used to study the influence of the diet-induced changes in the microbiota. In addition, stool microbiota of individuals with different types of habitual diets [e.g., vegetarians or vegans versus omnivores (9,10)] or from geographically distinct areas (11,12) have been characterized. It has become evident that the diet has a dominant role on the stool microbiota and that the diet-driven changes in it occur within days to weeks (8).

Dietary and physical activity patterns contribute to weight-imbalance disorders. Recently, there has been increased interest in the potential relation between gut microbiota and the development of obesity. Studies on energy-restricted diets administered in overweight and obese individuals aiming to relate the amount of body weight or weight loss to specific microbial groups have reported contradictory results (13–15). On the other hand, other studies involving human volunteers have mainly characterized the microbiota in stools according to individual BMI, regardless of diet (16–18). Previous work on obesity has shown distinct between-subject variations in stool bacterial diversity (13,19,20). Mixed genetic backgrounds and differences in analysis methodologies used (21) may, however,

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[3] Supplemental Tables 1–3 and Supplemental Figures 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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explain some of the controversies found in these studies. Studies in monozygotic (MZ) weight-concordant twins have shown that the MZ twins had a more similar stool bacterial community structure than unrelated individuals, suggesting a role for host genetic factors (18,22). It will therefore be very difficult to distinguish whether the changes observed in the stool microbiota of obese people are due to obesity itself, the diet related to it, or the host genetic make-up when obese and lean individuals from different genetic pools are compared. In contrast, MZ twins discordant for BMI offer an excellent tool for studying changes in the gut microbiota of obese and normal-weight subjects perfectly matched for genotype. Our aim was to study whether there is a correlation between the diet and the numbers and/or diversity of the predominant bacterial groups in stools. We studied the *Eubacterium rectale* group, the *Clostridium leptum* group, and *Bacteroides* spp., which together account for 50–70% of the human stool microbiota (11). Lactobacilli and bifidobacteria, although comprising smaller proportions of the stool microbiota, were also included, because both of those groups have traditionally been regarded as beneficial to human health. The *Atopobium* group (i.e., Coriobacteriaceae) has received less attention in the diet-related studies, but because it has been shown with hybridization-based studies that the *Atopobium* group comprises 1–5% of the stool microbiota (11) and that the proportion of Actinobacteria in stool microbiota may presently be underestimated (21,23,24), we also included the *Atopobium* group in our study. In addition to studying the correlation between the predominant bacterial groups and diet, we also studied how the intrapair differences in nutritional intake of MZ twins, concordant or discordant for BMI, correlated with the intrapair differences of bacterial group cell numbers and similarities.

Participants and Methods

Participants and sample collection. A total of 20 MZ twin pairs were recruited from a population-based longitudinal survey of 5 consecutive birth cohorts of twins (1975–1979) identified through the national population registry of Finland. The individuals were healthy based on their medical history and clinical examination, and their weight had been stable for at least 3 mo before the study. No medications other than contraceptives were used during the same period. Status of zygosity was confirmed by genotyping of 10 informative genetic markers (25). Weight, height, and body fat were measured as described previously (26). The participants were divided in categories according to their BMI (in kg/m^2) as follows: normal weight (19 ≤ BMI < 25), overweight (25 ≤ BMI < 30), and obese (BMI ≥ 30) (**Table 1**). Nine twin pairs were concordant (BMI difference <3 kg/m^2) and 11 pairs were discordant for BMI (BMI difference ≥3 kg/m^2); a BMI difference of 3 kg/m^2 represented the top 5% most discordant MZ twin pairs (25,27). Eleven of the twin pairs were female and 9 were male. The study protocols were approved by the ethics committee of the Hospital District of Helsinki and Uusimaa, Finland. Written informed consent was obtained from all participants. The participants collected the stool samples at home and stored them in their home freezer (−18°C) before taking the samples to the laboratory. The samples were stored in the laboratory at −70°C until analysis.

Nutritional intake. Dietary information was obtained from each individual based on a 3-d food diary (2 weekdays and 1 weekend day) that reflected the habitual dietary intake (**Table 2**). Although self-recorded estimates of food intake as food diaries may not provide accurate or unbiased estimates of a person's energy intake, the volunteers in our study were supervised by a specialist to ensure the best possible outcome. The food diaries were analyzed with the program DIET32 (Aivo Finland), which is based on a national database for food composition (28).

TABLE 1 Characteristics of the participants according to BMI group[1]

	Normal weight	Overweight	Obese
Age, *y*	26 ± 3	29 ± 3	28 ± 4
Height, *cm*	169 ± 10	170 ± 8.9	177 ± 11
Weight, *kg*	66 ± 11	79 ± 10	102 ± 16
Waist, *cm*	78 ± 6	88 ± 7	102 ± 10
Hip, *cm*	95 ± 7	101 ± 4.9	113 ± 5
Body fat, *kg*	19.2 ± 7.3	26.7 ± 5.3	41.5 ± 8.1
BMI, *kg/m^2*	22.9 ± 2.2	26.5 ± 1.2	32.4 ± 2.1

[1] Values are means ± SD. BMI groups (in kg/m^2): normal weight (19 ≤ BMI < 25), *n* = 11; overweight (25 ≤ BMI < 30), *n* = 18; obese (BMI ≥30); *n* = 11.

DNA extraction and qPCR of the stool samples. DNA extraction was performed from 0.2 g of stool sample as previously described (29). qPCR of "all" bacteria, *Bacteroides* spp., *E. rectale* group, *C. leptum* group, and *Atopobium* group in addition to bifidobacteria, was performed as previously described (21). qPCR of the *Lactobacillus* group was optimized and validated in the present study by using the primer pairs and bacteria listed (**Supplemental Tables 1** and **2**, respectively). qPCR amplifications of the *Lactobacillus* group were performed by using the High Resolution Melting Master Kit (Roche) with an adjustment of the $MgCl_2$ concentration of 3.0 mmol/L as follows: preincubation at 95°C for 10 min, an amplification step of 45 cycles of denaturing at 95°C for 15 s, primer annealing at 62°C for 20 s and elongation at 72°C for 25 s, a high-resolution melting step (95°C, 1 min; 40°C, 1 min; 65°C, 1 s; 95°C, 1 s), and cooling (40°C, 30 s).

Standard curves were obtained from genomic DNA templates isolated from pure cultures listed below. The extracted DNA was quantified by using NanoDrop 2000c equipment (Thermo Scientific). For each microorganism of interest, the number of cells present in the volume loaded to the qPCR reaction was calculated on the basis of the genome size and the respective 16S ribosomal RNA copy number per cell, identified through the National Center for Biotechnology Information genome database (30). A series of six 10-fold dilutions were performed per bacterial group qPCR by using the following type strains: *Anaerostipes caccae* VTT E-052773T (universal), *Bacteroides thetataiotaomicron* VTT E-022249 (*Bacteroides* spp.), *Roseburia intestinalis* VTT E-052785T (*E. rectale* group), *Anaerotruncus colihominis* VTT E-062942T (*C. leptum* group), *Bifidobacterium longum* VTT E-96664T (bifidobacteria), *Atopobium parvulum* E-052774T (*Atopobium* group), and *Lactobacillus casei* VTT E-58225T (*Lactobacillus* group). All qPCR reactions were performed by using

TABLE 2 Composition of the daily dietary intake of participants obtained from 3-d food diaries according to BMI group[1]

	Normal weight	Overweight	Obese
Energy, *MJ*	8.0 ± 1.7	8.4 ± 2.2	9.8 ± 2.0
Protein, *g*	85 ± 29	86 ± 31	81 ± 31
Total fat, *g*	77 ± 29	75 ± 26	85 ± 22
SFA, *g*	30 ± 12	28 ± 9.9	32 ± 8.6
MUFA, *g*	23 ± 8.9	19 ± 6.9	23 ± 6.9
PUFA, *g*	10 ± 4.0	10 ± 5.1	13 ± 5.2
(n-3) PUFA, *g*	1.8 ± 0.7	1.5 ± 0.7	1.6 ± 0.6
(n-6) PUFA, *g*	7.9 ± 3.2	8.6 ± 4.5	11 ± 4.4
Carbohydrates, *g*	200 ± 50	219 ± 58	255 ± 51
Sugars, *g*	86 ± 29	88 ± 36	129 ± 26
Starch, *g*	107 ± 39	106 ± 39	103 ± 40
Total fiber, *g*	21 ± 14	16 ± 6.1	17 ± 5.5
Soluble fiber, *g*	4.8 ± 3.6	3.8 ± 1.4	4.0 ± 1.5
Insoluble fiber, *g*	16 ± 12	11 ± 4.1	13 ± 3.9

[1] Values are means ± SD. BMI groups (in kg/m^2): normal weight (19 ≤ BMI < 25), *n* = 11; overweight (25 ≤ BMI < 30), *n* = 18; obese (BMI ≥30); *n* = 11.

the LightCycler 480 System (Roche), and the results were analyzed with the LightCycler 480 software version 1.5 (Roche).

Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene fragments. Denaturing gradient gel electrophoresis (DGGE) analysis of the predominant bacteria, *E. rectale* group, *C. leptum* group, *Bacteroides* spp., bifidobacteria, and *Lactobacillus* group, were performed as previously described (21,31). DGGE profiles were analyzed with BioNumerics software version 5.1 (Applied Maths BVBA). Clustering was performed with the Pearson correlation and the unweighted-pair group method by using an optimization of 0.5% and a position tolerance of 1.0%. Bands with a total surface area of ≥1% were included in the similarity analysis as previously described (31).

Statistical analysis. The study participants were considered both individually and as twin pairs. A linear mixed model was applied to study the effect of BMI and dietary intake of the individuals in the numbers of bacteria obtained by qPCR. The logarithm-transformed number of cells was modeled through linear relationships with the dietary quantitative variable where intercepts and slopes were assumed to depend on the combination of BMI and the bacterial group. The dietary variables considered were energy intake (kcal/d) and the following macronutrients (g/d): protein, SFA, MUFA, (n-3) PUFA, (n-6) PUFA, insoluble fiber, and soluble noncellulosic polysaccharides. These variables were considered as fixed effects in the model. Twin individuals and their families were treated as random effects to reflect the hierarchical structure of the data and accounting for interpair (between families) and intrapair (between co-twins) variation in the data. In addition, the residual variation was assumed to differ across the bacterial groups, and a general unstructured residual correlation assumption, which allows any correlation pattern, was included to reflect arbitrary dependencies between bacterial groups. The model assumptions were assessed for the initial model as described above. Subsequently, stepwise backward elimination of nonsignificant effects was performed by using likelihood ratio tests. For the resulting simplified model, pairwise comparisons of estimated mean intercepts and slopes between BMI groups within each bacterial group were performed with appropriate adjustment of P values for multiple testing.

The intra–twin pair difference in dietary intake (same nutrients mentioned above) measured between co-twins of the same family, BMI, and body fat were related with the intrapair difference of number of cells per bacterial group, the intrapair difference in diversity, and bacterial profile similarities between co-twins. General linear models were fitted for each individual bacterial group. Slopes of the regression lines were assumed to vary according to the concordance/ discordance status of the twin pair for BMI. Backward stepwise elimination of nonsignificant effects was performed by using likelihood ratio tests.

The comparison of the group-specific DGGE profiles between the co-twins was performed by calculating a similarity percentage. The intrapair similarities were divided into intervals for each bacterial group analyzed and correlated with the intrapair difference in dietary intake of the macronutrients mentioned previously. Similarity groups were labeled as "very low" (0–25% similarity), "low" (26–50% similarity), "high" (51–80% similarity), and "very high" (81–100% similarity). In the case of bifidobacteria, no similarity values were obtained above 80%. Mean differences between groups were evaluated by ANOVA.

The statistical environment R (32) was used for statistical analysis, in particular the R extension packages "nlme" and "multcomp" (33,34). P values <0.05 were considered to be significant.

Results

Association of nutritional intake with numbers of stool bacteria as studied with qPCR. The numbers of bacteria within the different bacterial groups, as measured by qPCR, did not differ between BMI groups (**Supplementary Fig. 1**; **Supplemental Table 3**). The association of the nutritional intake (all dietary components together) with the numbers of the different stool bacteria across the studied population was described through a hierarchical linear mixed model (**Fig. 1**). Intakes of energy, MUFA, (n-3) PUFA, (n-6) PUFA, and soluble fiber affected the numbers of the bacterial groups studied ($P < 0.01$). Individuals with high energy intake had significantly lower numbers of *Bacteroides* spp. ($P = 0.007$) and slightly higher numbers of bifidobacteria ($P = 0.02$) than did individuals with lower energy intake (Fig. 1*A*). The greater MUFA consumption was associated with lower bifidobacterial numbers ($P = 0.0005$) (Fig. 1*B*). Moreover, the increased ingestion of (n-3) PUFA had a significant association with higher numbers of bacteria within the *Lactobacillus* group ($P = 0.02$) (Fig. 1*C*). In contrast, greater (n-6) PUFA consumption was negatively correlated with the numbers of bifidobacteria ($P = 0.003$) (Fig. 1*D*). Soluble fiber intake had a positive association with the *Bacteroides* spp. numbers ($P = 0.009$) (Fig. 1*E*).

Association of nutritional intake with diversity and quantification of stool microbial groups within the twin pairs. The diversity of the studied bacterial groups, defined as the number of the bands obtained by different group-specific PCR-DGGE, did not differ between BMI groups (**Supplemental Fig. 2**). However, in co-twins with identical energy intake, the diversity of *Bacteroides* spp. as measured with group-specific DGGE was more similar than in co-twins with different intakes of energy ($P = 0.02$, $R^2 = 0.3$; data not shown). In addition, the co-twins with identical energy intake had more similar numbers of *Bacteroides* spp. than did the co-twins with different intake ($P = 0.03$, $R^2 = 0.3$; data not shown). No significant differences were obtained in the comparison of the numbers of bacteria within the other bacterial groups and diet. Moreover, no significant association was found between the intrapair difference in diversity or numbers of cells per gram of stool samples of the studied bacterial groups and concordance of co-twins for BMI, intrapair difference in BMI, or body fat.

Intra–twin pair similarities of DGGE microbiota profiles. The similarities (in %) of the bacterial group–specific DGGE profiles were calculated between co-twins. In addition, the difference in the amount of nutrients ingested was calculated within each twin pair and afterwards correlated with the DGGE similarities. Co-twins with the same SFA intake had very similar *Bacteroides* spp. profiles (80–100% similarity), which was significantly different from the twin pairs with distinct SFA intake (0–25% similarity; $P = 0.003$) (**Fig. 2***A*). The group of co-twins who daily consumed the same amount of fiber had very low bifidobacterial similarity (0–25%), which was significantly different from the group with high similarity (56–80%) ($P = 0.008$) (Fig. 2*B*). For the other bacterial groups studied by using DGGE, no significant associations were obtained regarding dietary intake. No relation was found between the intrapair DGGE-profile similarities and the co-twin concordance for BMI, intrapair difference in BMI, or body fat.

Discussion

The main environmental factors that affect the gut microbiota composition in generally healthy adults are diet and medication. The impact of the diet on the stool microbiota has usually been assessed by subjecting a group of individuals to the same controlled diet and consequently following the shifts in the microbiota. In the present study, the effect of habitual dietary intake in the stool microbiota of a population of Finnish MZ

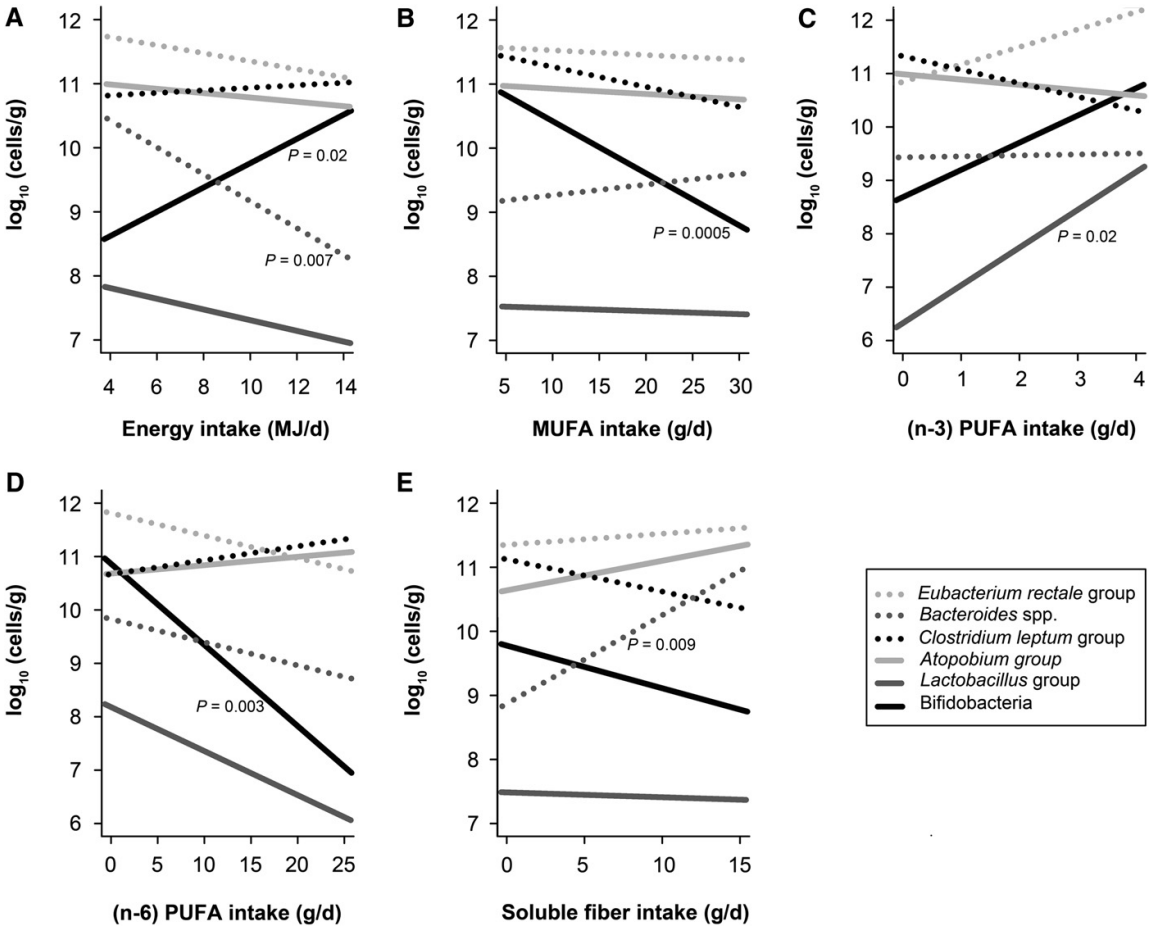

FIGURE 1 Association between the dietary intake of monozygotic twins and the number of cells of the stool bacterial groups, as measured by using group-specific qPCR; $n = 40$. Data are logarithm-transformed numbers of cells in the bacterial groups per gram of wet stool versus energy intake (*A*), MUFA intake (*B*), (n-3) PUFA intake (*C*), (n-6) PUFA intake (*D*), and soluble fiber intake (*E*).

twins was assessed through a hierarchical linear mixed model accounting for interpair and intrapair variations. No significant differences in the cell numbers of stool bacteria within the bacterial groups studied were observed between different BMI groups. In a longitudinal study in obese and lean twins, Turnbaugh et al. (18) reported a higher proportion of bacteria of the phylum Actinobacteria and lower proportion of the phylum Bacteroidetes in obese twins compared with lean twins, whereas no significant differences in the members of the phylum Firmicutes were observed. Although no difference in *Bacteroides* spp. numbers was detected in our study between BMI categories, the abundance of this bacterial group significantly decreased when the total energy intake increased. In addition, the co-twins of our study with similar daily energetic intake had more similar numbers and DGGE-profile diversities of *Bacteroides* spp. as compared with the twin pairs with different energy intakes. In accordance with our results, previous studies have also found a correlation between a low proportion of *Bacteroides*/*Prevotella* and high energy intake, rather than obesity (35). Moreover, according to Hildebrandt et al. (36), administration of a high-fat diet to both wild-type and *RELMβ* knockout mice, resistant to fat-induced obesity, increased the relative proportions of the phyla Proteobacteria, Firmicutes, and Actinobacteria in the feces, whereas the levels of Bacteroidetes decreased in both mice. This result indicated that the fat content in the diet itself rather than the obese state of the host induced the changes in microbiota composition. Although BMI is a validated measure of nutritional status, other physiologic, metabolic, and genetic factors, in addition to inadequate physical activity, are behind the etiology of the weight-balance disorders. Therefore, studies on the relationship between the gut microbiota and the host's health should not rely solely on BMI values but should also consider other variables such as diet composition.

Naturally occurring fats are mixtures of SFA, MUFA, and PUFA with one predominating type in most foods. Therefore, we also analyzed how the intake of different types of fats correlated with the stool microbiota composition. The high intake of MUFA was associated with lower numbers of bifidobacteria and slightly higher numbers of *Bacteroides* spp. In addition, the co-twins of our study who ingested identical levels of SFA had very similar *Bacteroides* spp. DGGE profiles (80–100%), suggesting that the intake of SFA affects the diversity of *Bacteroides* spp. by targeting specific strains within the same group. In a recent metagenomic study in healthy volunteers, the

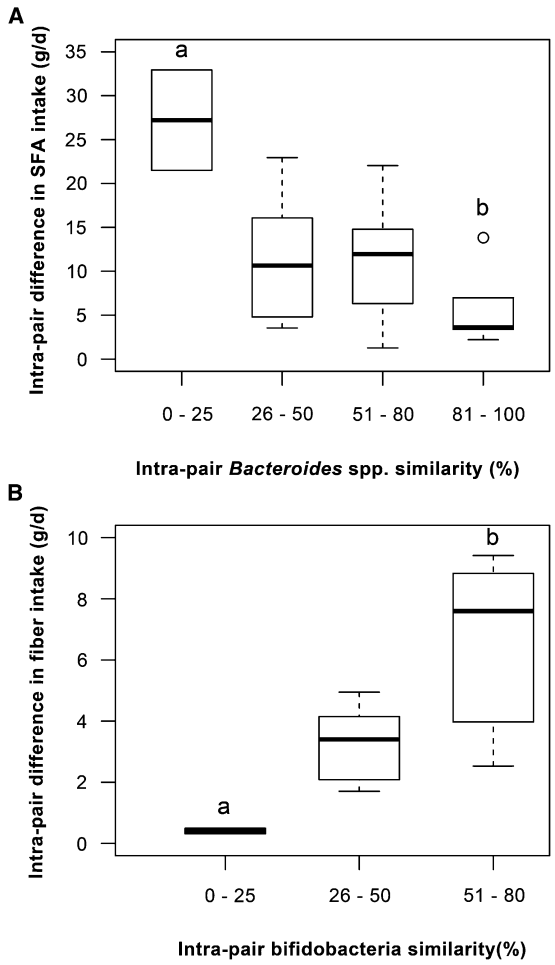

FIGURE 2 Association between the intra–twin pair difference in dietary intake and the intra–twin pair similarities as measured with bacterial group–specific denaturing gradient gel electrophoresis. *Bacteroides* spp. similarity versus intrapair difference in SFA intake (g), n = 5–7 twin pairs/group (*A*); bifidobacteria similarity versus intrapair difference in insoluble fiber intake, n = 4–7 twin pairs/group (*B*). Boxes show the medians (black line inside the box) and IQRs, and whiskers represent either the maximum and minimum values or 1.5 times the IQR of the data in case outliers are present. Circles outside the boxes represent outliers. Groups without a common letter differ, $P < 0.05$.

Bacteroides enterotype was found to be highly associated with the consumption of fat, in particular with mono- and saturated fat (37). These observations suggest that the consumption of fat and animal-derived products, typically present in the Western diet, are associated with increased *Bacteroides* spp. prevalence in the human gut microbiota. There are only a few studies in which the correlations between the types of dietary fat and the stool bacterial composition have been investigated. In our study, (n-3) PUFA intake resulted in a significant positive association with *Lactobacillus* group abundance. The increase in stool *Lactobacillus* group bacterial numbers after (n-3) PUFA intake was also previously reported in a mouse study (38). In addition, in a human study by Santacruz et al. (15), the numbers of lactobacilli remained at the same level, even though the ingested amount of total PUFA was greatly reduced. The increase in (n-3) PUFA has been reported to be effective in supporting epithelial barrier integrity by improving trans-epithelial resistance and by reducing IL-4–mediated permeability (39), and several lactobacilli have been shown to enhance the function of the intestinal barrier (40,41). Higher (n-6) PUFA intake was associated with decreased numbers of bifidobacteria in our study. This result is in agreement with previous studies, which reported that high (n-6) PUFA intakes decrease certain immune functions, such as antigen presentation, adhesion molecule expression, proinflammatory cytokines, and T-helper (Th) 1 and Th2 responses (42). Furthermore, genomic DNA of some bifidobacterial strains has been shown to stimulate the production of Th1 and proinflammatory cytokines, IFN-γ, and TNF-α (43). Overall, our results indicate an association between dietary fat types and their distinct effect on the fecal microbiota. As a consequence, it seems that balanced diet with regard to fat consumption is critical not only for the host's health but also for the gut microbiota.

Diet-derived carbohydrates are one of the main fermentative substrates of dietary origin in the colon and include plant cell wall polysaccharides (nonstarch polysaccharides), oligosaccharides, and resistant starch. These polymers arrive in various states of solubility, chain length, and association with other molecules (44). In the human colon, the microbiota metabolizes these indigestible fibers to short-chain fatty acids, composed mainly of acetic, propionic, and butyric acids, which have been implicated to have both local and systemic beneficial biological effects in the human body. Butyrate, for example, is the preferred fuel of colonocytes and also plays a major role in the regulation of cell proliferation and differentiation (45,46). In our study, the numbers of *Bacteroides* spp., *C. leptum* group bacteria, and *E. rectale* group bacteria were increased in individuals who consumed higher quantities of soluble fiber. Bacteria within the *Bacteroides* spp. have been recognized as versatile members of the dominant microbiota, carrying a vast array of polysaccharide hydrolyzing enzymes. Moreover, *Bacteroides* spp. bacteria have been associated with the use of soluble fibers (44). It has been reported in several studies that the high consumption of fiber is associated with an increased proportion of butyrate-producing bacteria (47,48). Because most of the butyrate-producing bacteria belong to the *C. leptum* and *E. rectale* groups, our results are in agreement with previous studies (49,50). In conclusion, our findings confirm that diet plays an important role in the modulation of the intestinal microbiota, in particular *Bacteroides* spp. and bifidobacteria.

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Online Supporting Material

Supplemental Table 1 Bacterial pure cultures used in this study for optimization of the *Lactobacillus* group qPCR

Species	Strain	Clostridial cluster [1]	Phylogenetic affiliation according to NCBI[2] taxonomy (Phylum, Family)
Anaerofilum agile	DSM 4272	IV	Firmicutes, Ruminococcaceae
Anaerofilum pentosovorans	DSM 7168	IV	Firmicutes, Ruminococcaceae
Anaerostipes caccae	VTT E-052773	XIV	Firmicutes, Lachnospiraceae
Anaerotruncus colihominis	VTT E-062942	IV	Firmicutes, Ruminococcaceae
Atopobium parvulum	VTT E-052774		Actinobacteria, Coriobacteriaceae
Bacteroides caccae	VTT E-062952		Bacteroidetes, Bacteroidaceae
Bacteroides fragilis	VTT E-022248		Bacteroidetes, Bacteroidaceae
Bacteroides ovatus	VTT E-062944		Bacteroidetes, Bacteroidaceae
Bacteroides thetaiotaomicron	VTT E-001738		Bacteroidetes, Bacteroidaceae
Bacteroides vulgatus	VTT E-001734		Bacteroidetes, Bacteroidaceae
Bifidobacterium adolescentis	VTT E-981074		Actinobacteria, Bifidobacteriaceae
Bifidobacterium breve	VTT E-981075		Actinobacteria, Bifidobacteriaceae
Bifidobacterium longum subsp. *longum*	VTT E-96664		Actinobacteria, Bifidobacteriaceae
Blautia coccoides	VTT E-052778	XIV	Firmicutes, Incertae Sedis XIV
Clostridium beijerinckii	VTT E-93498	I	Firmicutes, Clostridiaceae
Clostridium bolteae	VTT E-052776	XIV	Firmicutes, Lachnospiraceae
Clostridium butyricum	VTT E-97426	I	Firmicutes, Clostridiaceae
Clostridium clostridioforme	VTT E-052777	XIV	Firmicutes, Lachnospiraceae
Clostridium hathawayi	VTT E-062951	XIV	Firmicutes, Lachnospiraceae
Clostridium histolyticum	VTT E-052779	II	Firmicutes, Clostridiaceae
Clostridium indolis	VTT E-042445	XIV	Firmicutes, Lachnospiraceae
Clostridium leptum	VTT E-021850	IV	Firmicutes, Ruminococcaceae
Clostridium perfringens	VTT E-98861	I	Firmicutes, Clostridiaceae
Clostridium sporosphaeroides	VTT E-062947	IV	Firmicutes, Ruminococcaceae
Clostridium symbiosum	VTT E-981051	XIV	Firmicutes, Lachnospiraceae
Collinsella aerofaciens	VTT E-052787		Actinobacteria, Coriobacteriaceae
Desulfovibrio desulfuricans subsp. *desulfuricans*	VTT E-95573		Proteobacteria, Desulfovibrionaceae
Desulfovibrio vulgaris subsp. *vulgaris*	VTT E-95573		Proteobacteria, Desulfovibrionaceae
Dorea longicatena	VTT E-052788	XIV	Firmicutes, Lachnospiraceae
Eggerthella lenta	VTT E-001735		Actinobacteria, Coriobacteriaceae
Enterococcus faecalis	VTT E-93203		Firmicutes, Enterococcaceae
Enterococcus faecium	VTT E-93204		Firmicutes, Enterococcaceae
Escherichia coli	VTT E-94564		Proteobacteria, Enterobacteriaceae
Eubacterium eligens	VTT E-052844	XIV	Firmicutes, Lachnospiraceae
Eubacterium hallii	VTT E-052783	XIV	Firmicutes, Lachnospiraceae
Eubacterium ramulus	VTT E-052782	XIV	Firmicutes, Lachnospiraceae
Eubacterium siraeum	VTT E-062949	IV	Firmicutes, Ruminococcaceae
Faecalibacterium prausnitzii	DSM 17677	IV	Firmicutes, Ruminococcaceae
Fusobacterium necrophorum	VTT E-001739		Fusobacteria, Fusobacteriaceae
Lachnospira multipara	VTT E-052784	XIV	Firmicutes, Lachnospiraceae
Lactobacillus acidophilus	VTT E-96276		Firmicutes, Lactobacillaceae
Lactobacillus brevis	VTT E-91458		Firmicutes, Lactobacillaceae
Lactobacillus buchneri	VTT E-93445		Firmicutes, Lactobacillaceae
Lactobacillus casei	VTT E-85225		Firmicutes, Lactobacillaceae
Lactobacillus crispatus	VTT E-97819		Firmicutes, Lactobacillaceae
Lactobacillus fermentum	VTT E-93489		Firmicutes, Lactobacillaceae
Lactobacillus gasseri	VTT E-991245		Firmicutes, Lactobacillaceae
Lactobacillus johnsonii	VTT E-97851		Firmicutes, Lactobacillaceae
Lactobacillus paracasei	VTT E-93490		Firmicutes, Lactobacillaceae
Lactobacillus plantarum	VTT E-79098		Firmicutes, Lactobacillaceae
Lactobacillus reuteri	VTT E-92142T		Firmicutes, Lactobacillaceae
Lactobacillus rhamnosus	VTT E-97800		Firmicutes, Lactobacillaceae
Lactobacillus ruminis	VTT E-97852		Firmicutes, Lactobacillaceae
Lactobacillus salivarius	VTT E-97853		Firmicutes, Lactobacillaceae
Parabacteroides distasonis	VTT E-062943		Bacteroidetes, Porphyromonadaceae
Parabacteroides merdae	VTT E-062953		Bacteroidetes, Porphyromonadaceae
Prevotella melaninogenica	VTT E-052771		Bacteroidetes, Prevotellaceae
Roseburia intestinalis	VTT E-052785	XIV	Firmicutes, Lachnospiraceae
Subdoligranulum variabile	VTT E-062950	IV	Firmicutes, Ruminococcaceae
Veillonella parvula	VTT E-001737	IX	Firmicutes, Veillonellaceae

[1] Number of the clostridial phylogenetic cluster (1).
[2] National Center for Biotechnology Information, NCBI.

Online Supporting Material

Supplemental Table 2 Primers used in the present study

Target group	Probe / primer	Use	Sequence (5´ → 3´)	Reference
Predominant bacteria				
	U968-f +GC[1]	PCR-DGGE[3]	CGCCCGGGGCGCGCCCCGGGCGGGGCGGG GGCACGGGGGGAACGCGAAGAACCTTA	(2)
	U1401-r[1]	PCR-DGGE	CGGTGTGTACAAGACCC	(2)
	534R[2]	qPCR	ATTACCGCGGCTGCTGG	(3)
	358F[2]	qPCR	CCTACGGGAGGCAGCAG	(3)
Eubacterium rectale group[4]				
	Ccoc-f	PCR-DGGE	AAATGACGGTACCTGACTAA	(4)
	Ccoc-r + GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGGG GG CACGGGGGGCTTTGAGTTTCATTCTTGCGAA	(5)
	g-Ccoc-F	qPCR	AAATGACGGTACCTGACTAA	(6)
	g-Ccoc-R	qPCR	CTTTGAGTTTCATTCTTGCGAA	(6)
Clostridium leptum group[5]				
	Clept-933 f	PCR-DGGE	GCACAAGCAGTGGAGT	(6)
	Clept-1240-r+GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGGG GGCACGGGGGGGTTTTRTCAACGGCAGTC	(7)
	Clept-f	qPCR	GCACAAGCAGTCGAGT	(6)
	Clept-R3	qPCR	CTTCCTCCGTTTTGTCAA	(6)
Bacteroides spp.				
	Bact596f	PCR-DGGE	TCAGTTGTGAAAGTTTGCG	(8)
	Bacto1080r + GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGGG GGCACGGGGGGGCACTTAAGCCGACACCT	(7)
	g-Bfra-F	qPCR	ATAGCCTTTCGAAAGRAAGAT	(6)
	g-Bfra-R	qPCR	CCAGTATCAACTGCAATTTTA	(6)
Bifidobacteria				
	Bif164-f	PCR-DGGE	GGGTGGTAATGCCGGATG	(9)
	Bif662-GC-r	PCR-DGGE	CGCCCGCCGCGCGCGGCGGGCCGGGCGGG GGCACGGGGGGCCACCGTTAGACCGGGAA	(9)
	Bifid-f	qPCR	CTCCTGGAAACGGGTGG	(4)
	Bifid-r	qPCR	GGTGTTCTTCCCGATATCTACA	(4)
Atopobium group[6]				
	Atopo-f	qPCR	GGGTTGAGAGACCGACC	(6)
	Atopo-r	qPCR	CGGRGCTTCTTCTGCAGG	(6)
Lactobacillus group[7]				
	Lac1	PCR-DGGE	AGCAGTAGGGAATCTTCCA	(8)
	Lac2GC	PCR-DGGE	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCG CCCCCGCCCCATTYCACCGCTACACATG	(8)
	Lac1-F	qPCR	AGCAGTAGGGAATCTTCCA	(8)
	Lac2-R	qPCR	CATTYCACCGCTACACATG	(8)

[1] Partial 16S rRNA gene (V_6-V_8 hypervariable region)
[2] Partial 16S rRNA gene (V_3-V_5 hypervariable region)
[3] Denaturing Gradient Gel Electrophoresis, DGGE
[3] Clostridial phylogenetic clusters XIVa (1)
[4] Clostridial phylogenetic clusters IV (1)
[5] *Atopobium*-group comprises e.g. of genera *Atopobium*, *Eggerthella*, and *Collinsella*
[6] *Lactobacillus*-group comprises of the genera *Lactobacillus, Leuconostoc, Pediococcus*, and *Weissella*

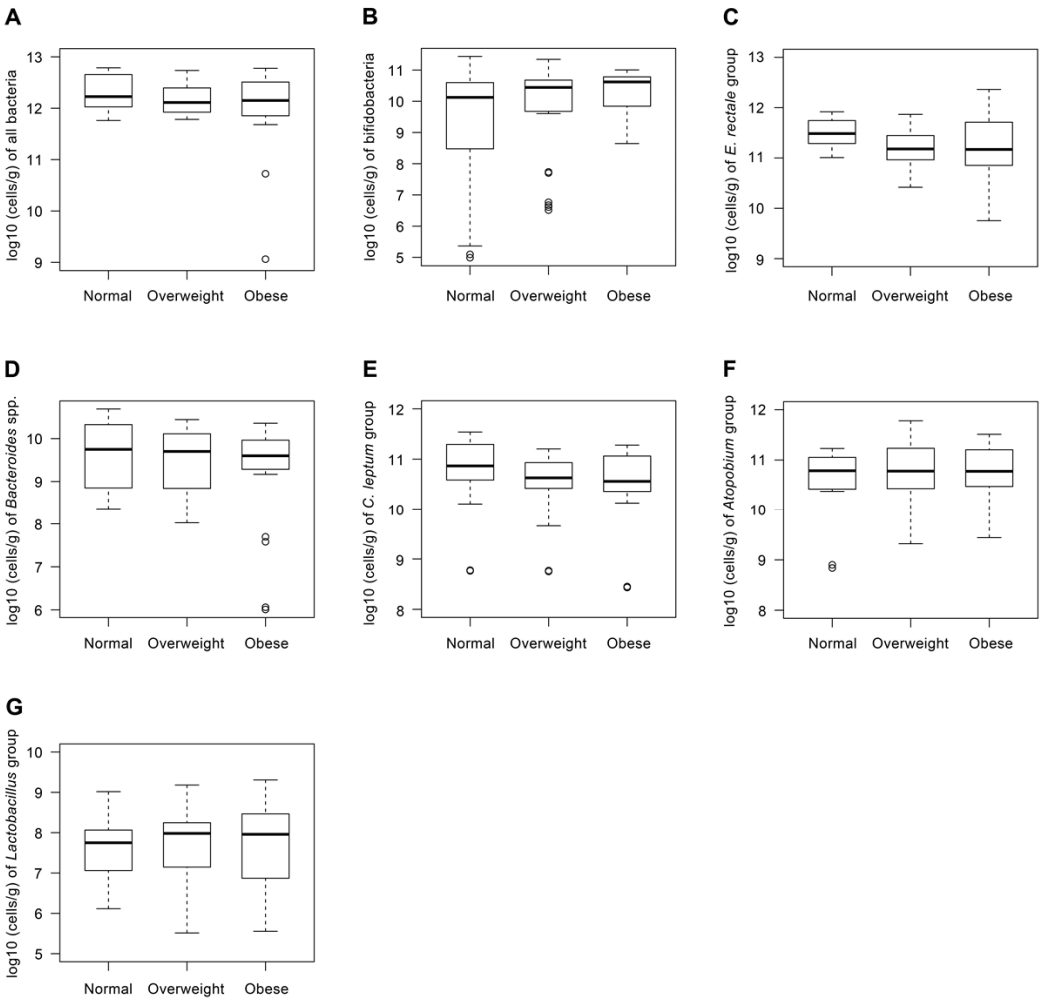
Online Supporting Material

Supplemental Table 3 Estimated mean differences of the number of cells per gram of wet stools between BMI groups, per bacterial group studied using qPCR[1]

Bacterial group	Overweight - Normal	Obese - Overweight	Obese - Normal
Atopobium group	-0.36 ± 0.27	0.18 ± 0.26	-0.18 ± 0.34
Bacteroides spp.	-0.06 ± 0.27	0.16 ± 0.26	0.09 ± 0.34
Bifidobacteria	0.09 ± 0.27	0.80 ± 0.26	0.89 ± 0.34
Clostridium leptum group	-0.40 ± 0.27	0.11 ± 0.26	-0.29 ± 0.34
Eubacterium rectale group	-0.37 ± 0.27	0.30 ± 0.26	-0.06 ± 0.34
Lactobacillus group	0.16 ± 0.27	0.28 ± 0.26	0.44 ± 0.34

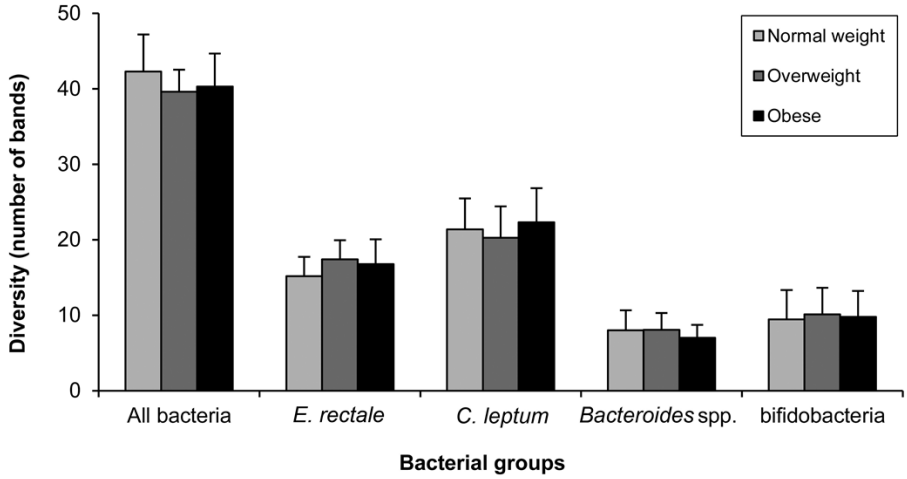
[1] Values are mean differences ± SEM of the logarithm transformed number of cells per gram of feces. SEM were obtained from the model output. BMI groups (in kg/m^2): Normal weight (19≤ BMI <25), *n* = 11; Overweight (25≤ BMI <30), *n* = 18; Obese (BMI ≥30), *n* = 11.

Online Supporting Material



Supplemental Figure 1 Number of cells of the stool microbiota as measured by qPCR, in the population of Finnish monozygotic twins grouped according to BMI. Data are logarithm-transformed number of cells per gram of wet stools of *all* bacteria (**A**), bifidobacteria (**B**), *Eubacterium rectale* group (**C**), *Bacteroides* spp. (**D**), *Clostridium leptum* group (**E**), *Atopobium* group (**F**), and *Lactobacillus* group (**G**). Box contains the median (black dot inside the box) and interquartile range, while whiskers represent either the maximum and minimum values or 1.5 times the interquartile range of the data in case outliers are present. Circles under the box represent the outliers. BMI groups (in kg/m^2): Normal weight (19≤ BMI <25), *n* = 11; Overweight (25≤ BMI <30), *n* = 18; Obese (BMI ≥30), *n* = 11.

Online Supporting Material



Supplemental Figure 2 Diversity of the stool microbiota measured by Denaturing Gradient Gel Electrophoresis in the population of Finnish monozygotic twins, grouped according to BMI. Stool bacterial groups: "all" bacteria, *Eubacterium rectale* group, *Clostridium leptum* group, *Bacteroides* spp., and bifidobacteria. Data are mean ± SD. BMI groups (in kg/m^2): Normal weight (19≤ BMI <25), *n* = 11; Overweight (25≤ BMI <30), *n* = 18; Obese (BMI ≥30), *n* = 11.

Online Supporting Material

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PUBLICATION III

Impact of a very low energy diet on the fecal microbiota of obese individuals

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Impact of a very low energy diet on the fecal microbiota of obese individuals

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Abstract

Purpose Study how the dietary intake affects the fecal microbiota of a group of obese individuals after a 6-week very low energy diet (VLED) and thereafter during a follow-up period of 5, 8, and 12 months. Additionally, we compared two different methods, fluorescent *in situ* hybridization (FISH) and real-time PCR (qPCR), for the quantification of fecal samples.

Methods 16 subjects participated in a 12-month dietary intervention which consisted of a VLED high in protein and low in carbohydrates followed by a personalized diet plan, combined with exercise and lifestyle counseling. Fecal samples were analyzed using qPCR, FISH, and denaturing gradient gel electrophoresis.

Results The VLED affected the fecal microbiota, in particular bifidobacteria that decreased approximately 2 logs compared to the baseline numbers. The change in numbers of the bacterial groups studied followed the dietary intake and not the weight variations during the 12-mo. intervention. Methanogens were detected in 56% of the participants at every sampling point, regardless of the dietary intake. Moreover, although absolute numbers of comparable bacterial groups were similar between FISH and qPCR measurements, relative proportions were higher according to FISH results.

Conclusions Changes in the fecal microbial numbers of obese individuals were primarily affected by the dietary intake rather than weight changes.

Keywords Obesity; Human fecal microbiota; Very low energy diet; Weight loss

Introduction

The balance between energy intake and energy expenditure is the basis of weight management throughout life. Energy intake is regulated by several mechanisms including neurotransmitters, hormones, body-fat storages, gut peptides, and post ingestion factors. On the other hand, energy expenditure is explained by the resting metabolic rate, the thermogenic effect of food, and the voluntary physical activity [1]. A longstanding positive energy balance results in overweight and obesity. Although abnormalities in any of the regulation mechanisms can lead to weight fluctuations, evidence strongly suggests that dietary and activity patterns are the primary causes of the weight gain [2]. Treatments based on energy-restriction diets, physical exercise and behavioral changes succeed to some extent to control obesity, although usually yield limited and transient weight loss. Therefore, the increased prevalence of obesity has brought new studies aiming at identifying environmental factors of the host that possibly affect the energy balance. Weight loss studies have found correlations between the caloric and nutrient intake and the fecal microbiota composition. A 10-week energy-restricted diet in adolescents increased counts of *Bacteroides fragilis* and decreased counts of *Blautia coccoides* and *Bifidobacterium longum* [3]. Similarly, after a 10-week energy-restricted diet obese adolescents had reduced counts of bacteria within the *Eubacterium rectale/Blautia coccoides* group and increased numbers of the *Bacteroides/Prevotella* group, which were associated with weigh loss above 4 kg [4]. Diet modification in combination with energy restriction is the most common treatment for moderate obesity. Very low energy diets (VLED) are defined as diets providing a maximum of 800 kcal with high quality protein [5]. Their major advantage is the rapid weight loss.

The aim of the present study was to investigate how the dietary intake affects the fecal microbiota of a group of obese individuals after a 6-week VLED, and thereafter during the follow-up period of 5, 8, and 12 mo. Moreover, we compared two different quantification methods, namely fluorescent *in situ* hybridization (FISH) and real-time PCR (qPCR). The numbers, diversity, and temporal stability (i.e., similarity) of selected bacterial groups were studied in order to determine correlations between changes in the dietary intake and fecal microbiota.

Materials and methods

Study participants and experimental design

A group of 16 Finnish obese subjects (BMI >30 kg/m^2; six males and 10 females; Table 1), participated in a 12-mo. study (Figure 1) , which consisted of individual and group-based weight loss treatment, together with exercise and lifestyle counseling. The study group consisted of healthy individuals, based on the medical history and clinical examination, and their weight had been stable for at least 3 months prior to the study. No medications other than contraceptives were used during the study period. Briefly, the energy-restricted treatment started with a 6-week modified VLED program high in protein and low in carbohydrates and fat. After the VLED, each participant followed a personalized diet plan adjusted to their personal needs until the end of the study, as previously described [6]. Dietary intakes described in Table 1 were obtained using validated individual 3-day food diaries (two weekdays and one weekend day) at the baseline of the study and at 5 and 12 mo., as previously described [7]. Food diaries were analyzed with the program DIET32 (Aivo, Finland), which is based on a national database for food composition [8]. All participants were supervised by the same nutritionist throughout the study. The participants collected the fecal samples at home and stored them in the home freezer (-20 ºC) before taking the samples to the laboratory. These frozen samples were stored in the laboratory at -80 ºC until analysis. The study protocols were approved by the ethical committee of the Hospital District of Helsinki and Uusimaa, Finland. Written informed consent was obtained from all participants.

Table 1. Characteristics and dietary intakes of the participants.

	Time				
	0 mo.	**1.5 mo.**	**5 mo.**	**8 mo.**	**12 mo.**
Weight (kg)	96.3 ± 13.3	87.1 ± 11.6	84.8 ± 12.8	84.2 ± 12.5	87.4 ± 14.7
Waist (cm)	110.6 ± 9.4	102.8 ± 9.1	98.3 ± 10.6	96.2 ± 9.3	97.9 ± 11.7
Fat mass (FM) (kg)	45.6 ± 17.3	n.a.	41.1 ± 8.9	n.a.	42.1 ± 9.5
Body mass index (BMI) (kg/m^2)	34.5 ± 2.6	31.3 ± 2.7	30.4 ± 3.2	30.4 ± 3.5	31.4 ± 4.1
Energy intake (kcal/ d)	2367 ± 973	800	1446 ± 452	n.a.	1744 ± 568
Carbohydrates (g/d)	281.6 ± 119.3	67	163.9 ± 51.6	n.a	196.5 ± 60.2
Protein (g/d)	94.1 ± 40.0	90	86.3 ± 32.0	n.a	96.5 ± 29.2
Fat (g/d)	89.3 ± 41.2	9.5	42.0 ± 17.9	n.a	59.1 ± 16.8
Fiber (g/d)	20.1 ± 9.1	22	23.0 ± 14.0	n.a	24.6 ± 8.9

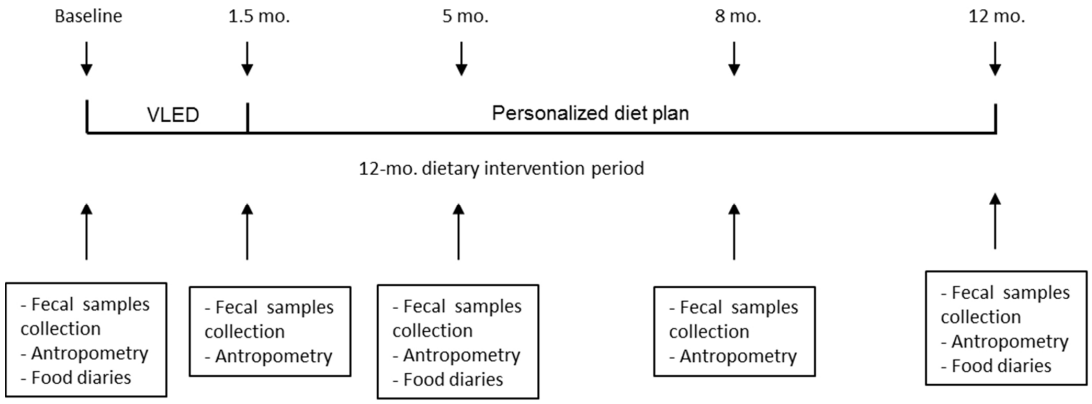

Figure 1. Study design.

DNA extraction and real time-Polymerase chain reaction (qPCR) of the fecal samples

DNA extraction was performed from 0.2 g of fecal sample as previously described [9]. qPCR of "all" bacteria, *Bacteroides* spp., *Eubacterium rectale* group, *Clostridium leptum* group, bifidobacteria, *Atopobium* group, and *Lactobacillus* group were performed as previously described [10]. qPCR of the methanogen group was optimized and validated in the present study using the primer pairs and microorganisms listed in the Supplementary Table 1 and Supplementary Table 2, respectively. Amplifications were performed using the High Resolution Melting Master Kit (Roche, Mannheim, Germany) with adjustment of the $MgCl_2$ concentration of 2.3 mmol/L. The protocol was as follows: pre-incubation step at 95 ºC for 10 min; amplification step consisted of 45 cycles of denaturing at 95 ºC for 10s, primer annealing at 62 ºC for 15 s, and elongation at 72 ºC for 20 s; high resolution melting (95 ºC 1 min, 40 ºC 1 min, 65 ºC 1 s, 95 ºC) and cooling (40 ºC, 30 s) steps. The standard curves for the absolute quantification of the target bacterial groups were created as previously described [10]. *Methanobrevibacter smithii* DSM 861 was used as a reference for the quantification of the methanogen group.

Table 2. Comparison of bacterial counts and relative abundances estimated using fluorescent *in situ* hybridization and qPCR in fecal samples of a subset of participants.

Bacterial group	FISH					qPCR				
	Probe	Bacterial counts (log_{10} cells/g)[1]		Relative abundance (%)[2]		Primers	Bacterial counts (log_{10} cells/g)[1]		Relative abundance (%)[2]	
		0 mo.	5 mo.	0 mo.	5 mo.		0 mo.	5 mo.	0 mo.	5 mo.
Universal	Eub338	10.2 ± 0.2	10.2 ± 0.4	-	-	358F/ 534R	11.6 ± 0.3	11.8 ± 0.4	-	-
Clostridial clusters XIVa + XIVb	Erec482	9.3 ± 0.4	9.3 ± 0.5	13.3	14.1	n.a.	n.a.	n.a.	n.a.	n.a.
Eubacterium rectale group (Clostridial cluster XIVa)	n.a.	n.a.	n.a.	n.a.	n.a.	Ccoc-f/ Ccoc-r	10.7 ± 0.6	10.6 ± 0.7	14.6	8.8
Bacteroides/*Prevotella*	Bac303	8.6 ± 1.0	8.9 ± 0.8	6.8	9.0	n.a.	n.a.	n.a.	n.a.	n.a.
Bacteroides spp.	n.a.	n.a.	n.a.	n.a.	n.a.	Bfra-f/ Bfra-r	8.9 ± 0.7	9.1 ± 1.2	0.3	0.6
Clostridium leptum group	n.a.	n.a.	n.a.	n.a.	n.a.	Clept-f/ Clept-r3	10.9 ± 0.4	10.7 ± 0.3	17.5	10.8
Ruminococcus[3]	Rbro730/ Rfla729	8.9 ± 0.5	9.1 ± 0.8	7.7	13.1	n.a.	n.a.	n.a.	n.a.	n.a.
Faecalibacterium prausnitzii[3]	Fprau645	9.2 ± 0.3	9.1 ± 0.4	13.1	12.3	n.a.	n.a.	n.a.	n.a.	n.a.
Roseburia/ E. rectale group[4]	Rrec584	8.9 ± 0.5	8.1 ± 0.4	4.8a	1.2b	n.a.	n.a.	n.a.	n.a.	n.a.
Clostridial cluster IX[5]	Prop853	9.4 ± 0.3	9.2 ± 0.5	15.6	12.6	n.a.	n.a.	n.a.	n.a.	n.a.
Atopobium group[6]	Ato291	8.9 ± 0.5	9.0 ± 0.5	7.5	7.7	Atopo-f/ Atopo-r	10.1 ± 0.4	10.1 ± 0.3	3.5	3.9
Bifidobacteria	Bif164	9.1 ± 0.7	8.9 ± 0.5	13.0a	6.3a	Bifid-f/ Bifid-r	9.4 ± 1.1	8.8 ± 1.4	1.4b	0.4b
Lactobacillus group	Lab158	7.5 ± 0.7	6.8 ± 0.6	0.4	0.07	Lac1-F/ Lac2-R	7.8 ± 0.8	7.4 ± 0.5	0.1	0.01
Eubacterium hallii	Ehal1464	7.3 ± 0.4	7.37 ± 0.5	0.12	0.3	n.a.	n.a.	n.a.	n.a.	n.a.

[1] Bacterial counts are expressed in log (cells/ g of wet feces) ± SD.

[2] The relative abundance of each bacterial group is given as percentage of the total bacterial count as measured by the universal probe Eub338; different letters differ, *P*-value < 0.05.

[3] Member of the *C. leptum* group.

[4] A subgroup within the Clostridial Cluster XIVa (*E. rectale* group), also detected with Erec482.

[5] Clostridial clusters according to Collins *et al.* [25].

[6] *Atopobium*-group comprises genera such as *Atopobium*, *Collinsella and Olsenella*

Enumeration of bacteria in fecal samples using fluorescent *in situ* hybridization (FISH)

Fecal samples (baseline and 5 mo.) from a subset of 8 participants were analyzed using FISH. Samples were prepared as previously described [11]. Briefly, diluted cell suspensions were applied to gelatine coated slides. Thereafter, the slides were hybridized overnight with 10 µl of the respective oligonucleotide probes and washed. Vectashield (Vector Laboratories, Burlingame, CA) was added to each slide to prevent fading. Fluorescing cells were visualized with a Leica DMRXA epifluorescence microscope. The total bacterial numbers were enumerated using the Eub338 probe, while specific bacterial groups were assessed using one of the probes in the panel (Supplementary Table 3).

Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene fragments

DGGE analysis of total bacteria, *E. rectale* group, *C. leptum* group, *Bacteroides* spp., and bifidobacteria were performed as previously described [12,13]. DGGE profiles were analyzed in terms of diversity and similarity between time points with the BioNumerics software version 5.1 (Applied Maths BVBA, Saint-Martens-Latem, Belgium) as previously described [13].

Statistical analysis

The logarithmically transformed numbers of cells of the bacterial groups studied were modeled through a linear mixed model used to account for repeated measurements in the same individual. The intercepts and slopes were assumed to depend on the combination of the variables time and the bacterial group, considered in the model as fixed effects. The individuals were treated as random effects, allowing for the variation between and within subjects. Pairwise comparisons of estimated mean intercepts between the different sampling points within each bacterial group were carried out with appropriate adjustment of *P*-values for multiple testing.

A paired *t*-test was used to evaluate the difference in bacterial numbers between the baseline and the 5 mo. samples. The paired *t*-test was used for both FISH and qPCR results.

The statistical analyses were carried out using the statistical environment R [14], in particular the R extensions packages "nlme" and "multcomp" [15,16]. $P < 0.05$ was regarded as statistically significant.

Results

Changes in the fecal bacterial numbers (measured using qPCR)

The predominant fecal bacterial groups of our 16 study subjects were quantified using qPCR. At the end of the VLED period, the total bacterial load, and the numbers of bacteria within the fecal bacterial groups studied tended to decrease, except that the numbers of *Bacteroides* spp. increased (Figure 2). The numbers of bifidobacteria had the most drastic reduction of approximately 2 logs at the end of the VLED as compared to the baseline ($P < 0.001$), followed by the *Lactobacillus* group with reduction of approximately 1 log. After the VLED period, the participants followed a personalized diet plan adjusted to their personal needs accompanied with individual supervision until 5 mo. At 5 mo., the fecal bacterial groups with the clearest changes were again bifidobacteria and the *Lactobacillus* group that increased 1.2 logs and 0.6 logs respectively compared to the VLED time point, although the increases were not significant. At 8 mo. the average weight loss of our volunteers had stabilized (Table 1), however, the numbers of bacteria within the bacterial groups studied tended to increase, except the *Bacteroides* spp. that stayed similar to the previous sampling point with approximately 9.5 log (cells/g of feces). At 12 mo., the numbers of *Bacteroides* spp. bacteria did not vary compared to 8 mo. time point, in contrast to the other fecal bacteria studied that decreased to numbers similar to those measured at 5 mo. At the end of the study, the weight measured from our volunteers was in average nine kg lower than at the baseline, and the daily energetic intake was approximately 630 kcal lower. Except *Bacteroides* spp., the other microbial groups studied using qPCR also had lower numbers at the end of the study as compared to the baseline. The methanogen group bacteria were detected from 56% of the study population. Each individual having methanogens, the presence of this group was detected in every sampling point. However, there was a very high inter-individual variation in the number of methanogen cells per gram of feces.

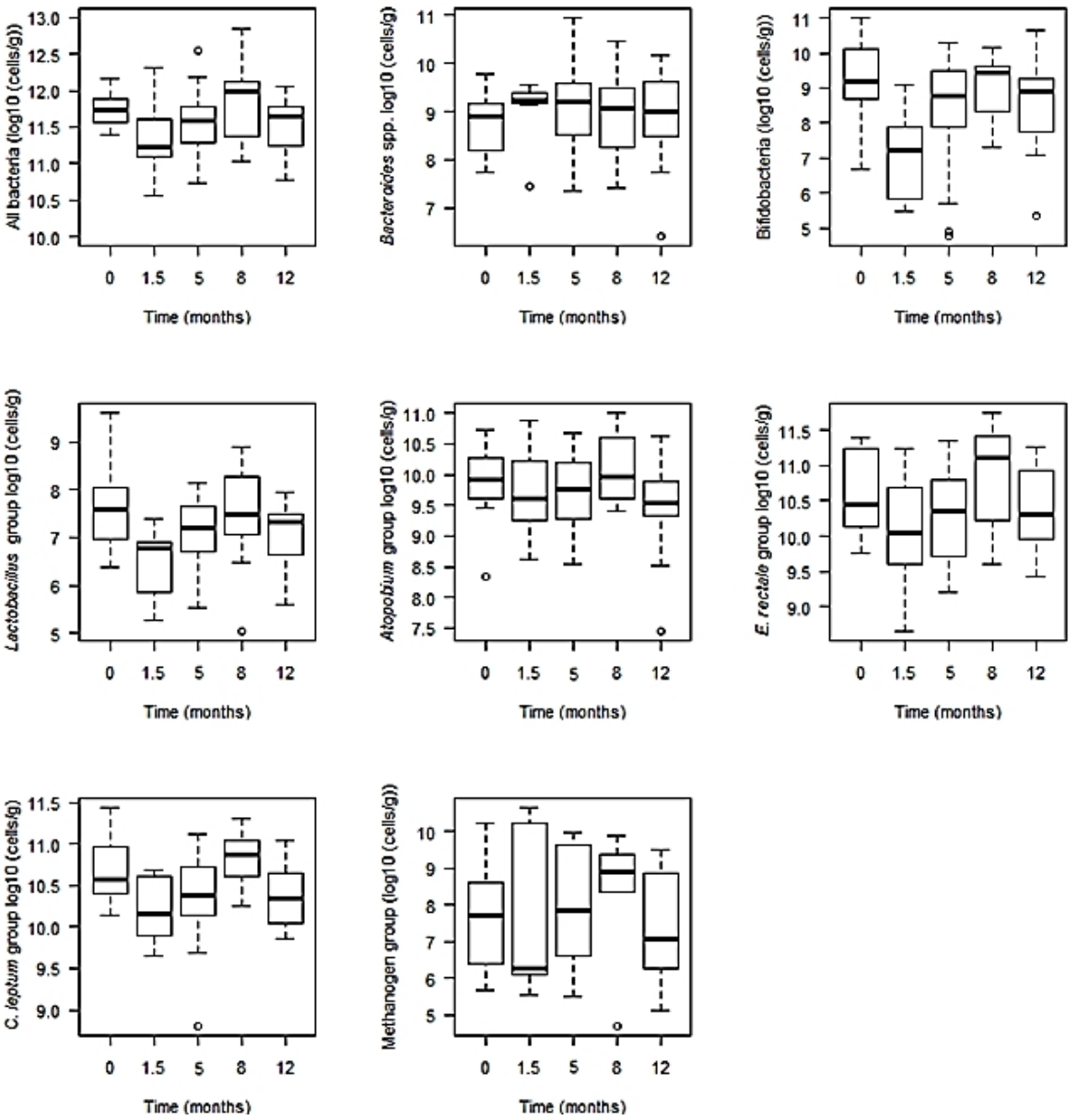

Figure 2. Quantification of bacteria within the studied fecal microbial groups as measured by qPCR in obese individuals during the course of a 12-months weight loss treatment. Box contains the median (black line inside the box) and interquartile range, while whiskers represent either the maximum and minimum values or 1.5 times the interquartile range of the data in case outliers are present. Circles outside the box represent the outliers.

Enumeration of the fecal bacteria using FISH and comparison to qPCR-based results

Fecal samples taken at baseline and 5 mo. were analyzed with FISH from a subset of 8 individuals, and the data compared to the results obtained with qPCR (Table 2). At the baseline, the most abundant fecal bacterial groups detected with FISH were the Clostridial clusters XIVa + XIVb, Clostridial cluster IX, *Faecalibacterium prauznitzii*, and bifidobacteria. At 5 mo., in addition to the Clostridial clusters XIVa + XIVb and Clostridial cluster IX, the most abundant groups were *Ruminococcus* and *F. prauznitzii*. The relative proportion of the *Roseburia/ E. rectale* group was significantly lower at 5 mo. (1.2%) as compared to the baseline (4.7%; $P = 0.046$). The same trend was observed with bifidobacteria (13% at 0 mo. vs. 6.3% at 5 mo.), however not statistically significant ($P = 0.07$). On the contrary, the abundance of the *Ruminococcus* group was higher at 5 mo. (13.1%) in comparison to the baseline of the study (7.7%; $P = 0.07$), although the *C. leptum* group (includes *Ruminococcus* genus) stayed similar as measured using qPCR. The numbers of bifidobacteria and the *Lactobacillus* group were similar at baseline and 5 mo. and when measured with FISH and qPCR. In addition, the number of bacteria within the *Atopobium* group was similar at both sampling points but one log higher when qPCR was applied as compared to the results obtained with FISH. Moreover, although the *Bacteroides-Prevotella* probe (Bac303) targets a wider range of species than the *Bacteroides* spp. qPCR primers (Bfra-f/Bfra-r), the bacterial counts observed were similar with the two methods and between sampling time points. On the other hand, although the FISH probe Erec482 targets the Clostridial clusters XVIa+b and the qPCR primers Ccoc-f/Ccoc-r only target the cluster XIVa, the absolute numbers were 1 log higher when measured with qPCR than with FISH.

The total bacterial numbers obtained with the universal qPCR primers at the baseline and 5 mo. (11.8±0.3 and 11.8±0.4 log (cells/g), respectively) were higher than the counts obtained with the universal FISH probe (10.2±0.2 and 10.2±0.4 log (cells/g), respectively). Therefore, the relative abundance of comparable bacterial groups was higher when measured with FISH than when using qPCR (Table 2). In particular, the relative abundance of bifidobacteria was significantly higher when measured with FISH than with qPCR ($P < 0.05$), even though the absolute numbers were similar in both sampling points.

VLED period: association with decreased energy intake and fecal bacterial numbers

The reduction in the energy intake between the VLED period and the baseline was correlated with the qPCR-based change in the numbers of bacteria. During the 6-week VLED period, the daily total energy intake decreased in average 64.6% [51.8% (min); 78.3% (max)]. The greater the energy reduction from the baseline, the lower the numbers of bifidobacteria measured at the end of the VLED period (P-value = 0.02; $R^2 = 0.6$). The change in the energy intake did not correlate with the change of the other bacterial numbers.

Diversity and temporal stability of selected bacterial groups

Bacterial group-specific DGGEs were used to assess the bacterial diversity during the weight loss intervention and the temporal stability, i.e., similarity of DGGE profiles of the groups studied between time points. The diversity of the total fecal bacteria, *E. rectale* group bacteria, *C. leptum* group bacteria, *Bacteroides* spp., and bifidobacteria stayed similar to the baseline diversities 29±4, 15±3, 17±4, 6±2, and 7±1 bands, respectively. However, amplification products were obtained from only 34% and 39% of the *Bacteroides* spp. and bifidobacteria specific PCRs, respectively, and there were no study subjects for whom all the samples amplified using these PCRs. Moreover, 65% of the 12 mo. samples amplified with the *Bacteroides* spp. specific PCR, whereas only 23–33% of the samples from the other sampling

time points amplified. In addition, 50–68% of the samples amplified with bifidobacteria specific PCR in every sampling point, except that at the 1.5 mo. sampling time point only one sample amplified. Therefore, the temporal stability of *Bacteroides* spp. population was not possible to obtain reliably. Temporal intra-individual similarity of total bacteria stayed rather stable between the sampling time points. However, the similarity values varied greatly between individuals (e.g. intra-individual similarity between baseline and 5 mo.: 34–87%). Intra-individual temporal stability of *E. rectale* group and *C. leptum* group tended to be higher between 8 and 12 months then between baseline and 5 months (Supplementary Figure 1).

Discussion

In the present study we report changes in the composition of the most abundant fecal bacterial groups during a 12-mo. energy-restricted diet intervention that included an initial 6-week VLED of 800 kcal/d, high in protein and low in carbohydrates and fat. During this period, the nutritional intake was similar for all the participants and corresponded to the period with the lowest daily energetic intake over the study, resulting in a decrease of body weight and thus BMI. The number of cells within the fecal microbial groups analyzed with qPCR tended to decrease during the VLED, except the *Bacteroides* spp. that slightly increased. Moreover, the numbers of bifidobacteria significantly decreased (~ 2 logs) after the 6-week diet followed by the *Lactobacillus* group. The low intake of carbohydrates may have reduced the substrates available for bifidobacteria and bacteria within the *Lactobacillus* group in the large intestine. Furthermore, the decrease of bifidobacteria and *Lactobacillus* group bacteria during the VLED possibly reflect the low intake of n-3 polyunsaturated fatty acids (n-3 PUFA). A maximum of 0.5 g per day of α-linolenic acid is recommended by the SCOOP task report of the European commission for VLED programs. The intake of such low amounts of n-3 PUFA has been associated with lower abundance of bifidobacteria and bacteria within and *Lactobacillus* group in fecal samples of Finnish monozygotic twins [10]. The same study associated the increase of soluble fiber intake with higher numbers of *Bacteroides* spp. and lower numbers of bifidobacteria. These results are consistent with the present study since the proportion of fiber during the VLED intervention was high as compared to the baseline. On the other hand, the high content of protein in the VLED may have contributed to the increase of *Bacteroides* spp. bacteria, the predominant proteolytic species identified in the human large intestine [17]. The increase of *Bacteroides* spp. during energy-restricted diets has been previously reported [4,3], however in these studies the reduction of total energy intake was not as drastic as during our VLED intervention. On the contrary, longitudinal studies involving severely obese patients subjected to a gastric bypass are more comparable, since the gastric bypass results in a drastic reduction of food consumption in a short period of time, however not necessarily low in carbohydrates. A recent study reported an increase in the numbers of *Bacteroides/Prevotella* group bacteria 3 mo. after a Roux-en-Y bypass surgery, while the numbers of bacteria within the *Bifidobacterium* and *Lactobacillus/Leuconostoc/Pediococcus* groups significantly decreased [18]. These results are consistent with our study.

After the VLED period the participants followed a personalized diet plan supervised until month five of the study and thereafter continued without supervision until 12 mo. At 5 mo., the total energy intake was higher than during the VLED (~ 1500 vs. 800 kcal/day, respectively), while BMI decreased reaching its lowest value during the study (30.4 ± 3.2 kg/m^2). The changes in the numbers of bacterial groups studied followed the change in dietary intake and not that of the BMI. At 5 mo., the relative abundance of the *Roseburia/ E. rectale* group as measured using FISH was significantly lower than at the baseline. In a previous study, obese individuals undergoing both moderate carbohydrate and low carbohydrate weight loss diets in alternate orders, had a decrease in the numbers of bacteria within the *Roseburia/E. rectale* group during both diets [19]. Moreover, the proportion of bifidobacteria

significantly decreased in subjects following a 4-week weight loss treatment on both of the above-mentioned diets. These results are in accordance to our study since the VLED intervention also had low carbohydrate content. Our study confirms that the alteration of the nutrient load induces changes in the gut microbiota abundance, as suggested before [20].

The methanogen group was detected in 56 % of the study population using qPCR. For each individual having methanogens, the presence of this group was detected in every sampling point, with the exception of one point from one individual, regardless of the weight loss or weight gain. This observation suggest that the presence of methanogens is host-specific as alluded before [21]. Although the median number of methanogens per gram of feces decreased at the end of the VLED and increased with the return to the conventional diet, the inter-individual variation among the participants was large. In a previous study, the number of copies of *M. smithii* species was found to be significantly higher in anorexic patients than in lean controls [22]. Methanogens have been shown to increase the efficiency of bacterial fermentation in the colon by utilizing the end product H_2 [23], therefore enhancing the fermentation of food materials in very low caloric diets [22].

Fecal samples of a subset of 8 participants (baseline and 5 mo.) were analyzed using both FISH and qPCR. The numbers of the comparable bacterial groups showed similar trends with both methods. However, although the absolute numbers obtained were close between the two methods, their relative abundance, calculated as percentage of the total bacteria counts, was higher when measured with FISH than with qPCR since the numbers of total bacteria targeted with the qPCR primers were almost 2 logs higher than the FISH counts. These observations indicate that caution is necessary when considering relative proportions of fecal bacterial groups obtained with different methods. In addition, the use of different primers or probes targeting similar groups of bacteria within the fecal microbiota should be taken into consideration, especially in the case of Bacteroidetes [24].

The diversity of the bacterial groups studied with PCR-DGGE stayed stable during the time studied. It has been shown previously that bacterial numbers do not necessarily correlate with bacterial diversity [9] and therefore our results are not contradictory to each other. It is however surprising that the variation in dietary intake did not affect the bacterial diversity as measured with DGGE, suggesting a strong host effect on the composition of the fecal bacterial population.

Our findings confirm that the dietary intake modulates the abundance of the predominant fecal microbial groups. Drastic variations in dietary intake occurred mainly during the VLED and thereafter with the return to the conventional diet. Except *Bacteroides* spp., the numbers of the other microbial groups analyzed decreased during the VLED, in particular bifidobacteria. In the end of our 12-mo. intervention, the microbial groups studied had similar numbers to those measured at 5 mo. reflecting that the change in fecal microbial numbers are associated with the dietary intake rather than the body weight variations.

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Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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PUBLICATION IV

Expression of *clpL*1 and *clpL*2 genes in *Lactobacillus rhamnosus* VTT E-97800 after exposure to acid and heat treatments or to freeze-drying

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Expression of *clpL1* and *clpL2* genes in *Lactobacillus rhamnosus* VTT E-97800 after exposure to acid and heat stress treatments or to freeze-drying

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Abstract

The aim of the study was to evaluate the potential of utilising the information on expression levels of selected stress genes in assessing the quality of probiotic products. For this purpose RT-qPCR methods were developed to study the expression of *clpL1* and *clpL2* stress genes in *Lactobacillus rhamnosus* VTT E-97800 (E800) cells after exposure to processing-related stress conditions or to freeze-drying. Heat treatments in laboratory scale were performed with E800 cells incubated at 47 °C or 50 °C for 60 min. Acid treatments were performed both at laboratory and fermenter scale. At laboratory scale E800 cells were inoculated into General Edible Medium (GEM) adjusted to pH 4.0 and pH 3.5 and incubated at 37 °C for 180 min, whereas fermenter-grown cells were exposed to pH 4.0 for 60 min at the end of the fermentation. RNA from fresh cells and freeze-dried powders was reverse transcribed after isolation, quantification and standardisation. *clpL1* and *clpL2* transcripts were analysed by RT-qPCR with SYBR Green I. *clpL1* was induced in *L. rhamnosus* E800 cells exposed to 50 °C and to a much lesser extent to 47 °C. No induction was observed for *clpL2* in E800 cells during either acid or heat treatment, in any of the conditions applied. RNA isolation from freeze-dried powders was unsuccessful although several attempts were made with high quality products. In conclusion, our results suggest that developing quality indicators for probiotic products based on differences in the expression of stress genes is a challenging task for several reasons: at least with some genes (like in the present study with *clpL*) quite harsh conditions are needed to detect differences in the gene expression; mRNA isolation from freeze-dried powders was unsuccessful which hampers the quality analysis of large proportion of probiotic products; and furthermore RT-qPCR proved to be a too laborious procedure for routine use.

Keywords: *Lactobacillus rhamnosus*, stress response, fermentation

1. Introduction

Adaptation of microorganisms to adverse environmental conditions typically induces a metabolic stress response consisting of changes in the gene expression pattern (Aertsen and Michiels, 2004; Pichereau *et al.*, 2000). Probiotic bacteria encounter stressful conditions e.g. during production and processing into foods, in storage, and subsequently in the digestive tract (Saarela *et al.*, 2004; Van de Guchte *et al.*, 2002). As in other bacteria, adaptive responses mediated by molecular chaperones and proteases appear to be one of the major means of stress protection in lactic acid bacteria. However, the molecular basis of such responses to each stress is partially species specific (Sugimoto *et al.*, 2008). The ATP-dependent Clp (proteinase) proteins, which include ClpL as member of the Clp/Hsp100 ATPase family, play an important role during the stress adaptation mechanism (Suokko *et al.*, 2005). Two encoding genes of ClpL proteins, *clpL1* and *clpL2*, have been identified in the *Lactobacillus rhamnosus* VTT E-97800 (E800) genome (Suokko *et al.*, 2005). At the moment there is no evidence of other *Lactobacillus* strains with multiple *clpL* genes. *clpL* genes have been shown to be induced by high temperatures in several *Lactobacillus* species and also in *Streptococcus thermophilus* (Suokko *et al.*, 2008; Varcamonti *et al.*, 2006).

The aim of the study was to evaluate the potential of utilising the information on the expression levels of selected stress genes in assessing the quality of probiotic products. Gene expression studies could potentially provide additional useful information on the physiology of cells, and thus the quality of probiotic products, besides more traditionally used culture-based or viability stain-based studies. For this purpose RT-qPCR methods were developed to study the expression of *clpL1* and *clpL2* stress genes in *L. rhamnosus* VTT E-97800 (E800) cells after exposure to processing-related stress conditions or to freeze-drying.

2. Material and methods

Bacterial strain

L. rhamnosus VTT E800 was obtained from the VTT culture collection (Espoo, Finland). The strain was stored at -70 °C and revived on De Man-Rogosa-Sharpe (MRS; Oxoid, Basingstoke, UK) at 37 °C anaerobically (H_2/CO_2/N_2, 10/10/80%; Anoxomat, MART, Lichtenvoorde, the Netherlands).

Stress conditions

Acid and heat stresses were applied in the present study since they both can be encountered during the production stages of probiotic products. Acid stress treatments were performed in laboratory and fermenter scale, whereas heat stress was studied only at laboratory scale.

At laboratory scale *L. rhamnosus* E800 cells were grown in 200 ml of General Edible Medium (GEM; Saarela *et al.*, 2004) at 37 °C until the stationary phase (18 h) was reached. Cells were divided into aliquots of 5 ml and centrifuged. For heat stress, cells were resuspended into the same volume of GEM. Triplicates were incubated at 37 °C (control), 47 °C and 50˚C. Samples were collected for viability study and RNA isolation after 10, 30 and 60 min. For acid stress cells were resuspended in triplicate into 5 ml of GEM adjusted to pH 4.0 and pH 3.5 with 1 M HCl, and incubated at 37 °C. Non pH adjusted GEM (pH 6.0±0.2) was used as a control. Samples were collected for viability determination by culturing on MRS agar, pH measurement, and total RNA isolation after 30, 60 and 180 min incubation.

For the fermenter scale acid stress *L. rhamnosus* E800 was grown in 5 l of GEM in the fermenter (Biostat® CT, B. Braun Biotech International, Melsungen, Germany). Fermentation was performed under controlled temperature of 37 °C and pH of 5.8 by addition of 25% NH_4OH (Merck, Darmstadt, Germany), constant stirring of 100 rev/min and under nitrogen flux. After reaching stationary phase (21 h), cells were stressed by addition of 18.5% HCl (Riedel de Haën, Seelze, Germany) until pH reached 4.0. Samples were collected for viability determination by culturing on MRS agar and total RNA isolation at the end of fermentation (control), and after 30 and 60 min of acid stress treatment.

Freeze-dried cells

L. rhamnosus E800 freeze-dried cells were prepared in two batches according to Saarela *et al.* (2006). Briefly, *L. rhamnosus* E800 was grown in 10 l of GEM for 20 h under pH control, neutralised with NaOH and centrifuged. Cell concentrates were mixed with the carrier polydextrose (10% w/v) and after 1 h incubation at room temperature the cell-carrier pellets were freeze-dried with a standard programme with an Epsilon 2-25 freeze-dryer (Martin Christ, Duingen, Germany). Freeze-dried powders were packed into aluminium foil sachets and stored at -20 °C for 24 months. The viability and stability of the powders was studied by culturing on MRS agar.

Molecular detection of *clpL1* and *clpL2* in *Lactobacillus rhamnosus* E800

The PCR amplification of *clpL1* in *L. rhamnosus* E800 was performed using the forward primer 5´-TTTCTCGAGT TGCTTTATCAGATGGTTGAGC (p14) and the reverse primer 5´-TTGGTACCATTATTCTTCGTCCGCCG (p15), according to Suokko *et al.* (2005). The same author was contacted in order to obtain the correct primers for the amplification of *clpL2*. The forward primer used was 5´-TCTCGAGAGGATATTATGGTCACTAAGTTACAC (p16) and the reverse primer 5´-TTAAGCTTTG CTTTAACTTCCTTTACCAGCTG (p17).

DNA was extracted using the FastDNA Spin Kit for soil (QBIOgene, Carlsbad, CA, USA) according to the manufacturer's instructions except that the bacterial cells were lysed with a Fast Prep instrument (Bio 101 Savant, Holbrook, NY, USA) at 6.0 m/s for 45 s three times. The isolated DNA was stored at -20 °C.

Optimised PCR amplifications were performed in a total volume of 20 µl. The reaction mixture for the amplification of *clpL1* contained 1 µl of DNA template, 0.4 µl of both primers, 0.16 µl dNTP, 0.1 units of Taq polymerase (Invitrogen, Carlsbad, CA, USA) and 1.1 µl $MgCl_2$. For *clpL2* the reaction was composed by 1 µl of DNA template, 0.4 µl of both primers, 0.2 µl dNTP, 0.1 units of Taq polymerase and 0.8 µl $MgCl_2$. Before the amplification, the reaction mixtures were incubated at 95 °C for 5 min. The PCR conditions for *clpL1* were 30 cycles of amplification consisting of denaturation at 95 °C for 45 s, annealing at 59 °C for 45 s, and extension at 72 °C for 60 s, followed by one cycle at 72 °C for 10 min. *clpL2* was amplified during 30 cycles consisting of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 60 s, followed by one cycle at 72 °C for 10 min.

RNA extraction

Total RNA was extracted and purified by using RNeasy Protect Bacteria Mini Kit (Qiagen, Hilden, Germany) in conjunction with the On-column DNase Digestion with the RNase-Free DNAse Set (Qiagen). One millilitre of each sample from the stress experiments was treated with RNAprotect Bacteria Reagent (Qiagen) according to the manufacturer´s instructions. Freeze-dried cells were first dissolved in 25 ml of peptone saline solution and diluted 1:10 and 1:100. 1 ml of each sample was treated with RNAprotect Bacteria Reagent according to the manufacturer's instructions. Cells were frozen at -80 °C until examined.

After thawing the stabilised cells, 350 µl of Buffer RTL (Qiagen) with β-mercaptoethanol (10 µl/1 ml RTL) was added per sample. Mixture was vortexed for 5-10 s and transferred to 2 ml Fast-Prep tubes containing acid-washed glass beads (150-212 microns Sigma, Steinheim, Germany). Cells were lysed in a FastPrep FP120 Instrument (Bio 101 Savant) for 45 s at 6.5 m/s three times. Samples were centrifuged at maximum speed during 1.5 min and aqueous upper part was transferred into a new tube to which an equal volume of ethanol 70% was added. Lysates were then transferred to RNeasy Mini spin columns placed in a 2 ml collection tube and manufacture's instructions were followed from this point onwards. The concentration and purity of the total RNA was analysed with a Biophotometer 6131 (Eppendorf, Hamburg, Germany).

Reverse transcription

Total RNA was reverse-transcribed by using Quantitect Reverse Transcription Kit (Qiagen). The volume of RNA template to be used during the reaction was standardised to150 ng/µl. This value was optimised within a range of 20 to 300 ng/µl of RNA template by comparing the posterior real time-PCR results. Reverse transcription master mix and reaction were performed according to the manufacturer's instructions.

Real time quantitative PCR

Real time quantitative PCR (RT-qPCR) amplification reactions were performed in the LightCycler Carousel-Based System (Roche, Mannheim, Germany), using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche). The reaction mixture (20 µl) contained 1 µl cDNA template, 2 µl LightCycler FastStart DNA Master SYBR Green I 10 x, 2.4 µl $MgCl_2$ (25 µM), 1 µl forward and reverse primer (5 µM *clpL1* and 10 µM *clpL2*) and 11.6 µl H_2O, PCR-grade. Before amplification, each reaction mixture was transferred to a capillar and then denaturated at 95 °C for 10 min. The amplification profile was as follows for *clpL1*: 50 cycles of 95 °C for 10 s, 61 °C for 10 s and 72 °C for 20 s. *clpL2* amplification profile was: 50 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 20 s. The specificity of all the reactions was confirmed through analysis of the melting profile, obtained by dissociation of the DNA after amplification.

The effect of the various stress treatments in the expression of *clpL1* and *clpL2* in *L. rhamnosus* E800 cells was screened and the corresponding crossing point (ct) values obtained. Data were analysed using the LightCycler Software 3.5 (Roche). The difference between the average of ct values obtained from the controls and the average of ct values from the stressed samples was calculated and the results compared for each sampling point. At the time 0 min, the difference was considered to be 0 since sample and control cells were obtained from the same batch of growth.

Statistical analysis

The mean and standard deviation were calculated for each experiment. Student's t-test with two tail assuming unequal variances was used for the statistical analysis of the results.

3. Results

The stress conditions for the present study were chosen so that they would not reduce the viability as determined by culture of the probiotic cells. Thus in the present study no decline in viability was seen for *L. rhamnosus* E800 cells after the heat or acid treatments performed (data now shown).

clpL1 was induced in *L. rhamnosus* E800 cells exposed to 50 °C and to a much lesser extent in cells exposed to 47 °C (Figure 1). At 47 °C the expression of *clpL1* had a slight decrease between the 10 min and 30 min of exposure, being constant after 30 min until the end of the treatment with a difference of approximately 2 cycles to the control. The same trend was observed at 50 °C during the first 30 min of exposure. However, an increase in the expression was observed after 30 min, with a difference of 5.7 cycles to the control after 60 min. Results were confirmed for two additional tests with 60 min samples (data not shown). No induction of *clpL2* was seen during the heat treatments (data not shown). Acid treatments did neither induce the expression of *clpL1* nor that of *clpL2* (data not shown). Melting curve analysis showed that the PCR products of all the stressed samples were specific.

In freeze-dried samples no amplification was observed for *clpL1* and *clpL2* transcripts, confirmed by the melting curve analysis. Repeated attempts to isolate good quality or quantity mRNA from freeze-dried *L. rhamnosus* E800 powders were unsuccessful. The quality of the powders was good (culture-based viability was 1×10^{11} cfu/g), so loss of viability does not explain this result.

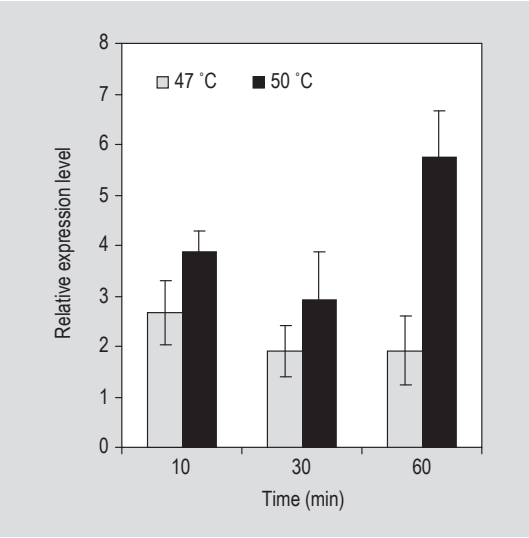

Figure 1. Relative expression level (Δ ($ct_{control}$ – ct_{sample})), measured by RT-qPCR, of *clpL1* of stationary phase *Lactobacillus rhamnosus* E800 cells subjected to heat stress treatment at 47 °C and 50 °C. The results were statistically significant (*P*<0.05) for all the measurements.

4. Discussion

In our previous study on *Bifidobacterium animalis* subs. *lactis* Bb-12 freeze-dried cells (Mättö *et al.*, 2006) we showed that a decrease in cell functionality (in this case tolerance to low pH) was more prominent than decrease in viability during storage, indicating that studying only viability/stability is not enough to predict the quality of probiotic products. Therefore, in the present study, we wanted to evaluate the potential of using information about the stress gene expression levels to facilitate in the quality assessment of probiotic products. For this study we chose *L. rhmanosus* VTT E-97800 strain with two well characterised stress genes, *clpL1* and *clpL2* (Suokko *et al.*, 2005).

In the present study we evaluated the expression of *clpL1* and *clpL2* by comparing the crossing point values obtained from RT-qPCR in samples subjected to different sub-lethal conditions that probiotics might potentially encounter during processing. According to RT-qPCR analysis, the expression of *clpL1* in *L. rhamnosus* E800 stationary phase cells was induced by sub-lethal heat treatments. In contrast, *clpL2* seemed not to be affected by heat stress at 47 °C or at 50 °C in *L. rhamnosus* E800. At 50 °C *clpL1* was more pronouncedly expressed as compared to 47 °C, especially after a 60 min exposure. Northern blot analysis carried out by Suokko *et al.* (2005) revealed that *clpL1* expression was strongly induced in *L. rhamnosus* E800 exponential phase cells subjected to 50 °C compared to *clpL2* transcripts which were only very moderately induced (Suokko *et al.*, 2005). The results of our study confirm those of Suokko *et al.* (2005) that the *clpL1* gene is involved with the adaptation mechanism of *L. rhamnosus* E800 cells exposed to sub-lethal heat stress conditions. The failure in the present study to repeat the anticipated results on low level of *clpL2* induction can probably be explained by methodological differences in the two studies and also by the differences in the physiology of the cells.

Acid stress of stationary phase *L. rhamnosus* E800 cells in moderate conditions (at pH 4.0 or pH 3.5 for up to 3 h) did not induce *clpL1* or *clpL2* expression in the present study. In *Oenococcus oeni* ATCC BAA 1163 growth in a special medium at pH 3.5 induced the expression of *clpL1* about 12-fold and *clpL2* four- to six-fold when mRNA levels were measured by RT-qPCR (Beltramo *et al.*, 2006). In the study of Wall *et al.* (2007) late exponential phase cells of *Lactobacillus reuteri* ATCC 55730 showed an induction of *clpL* after a transfer from pH 5.1 to pH 2.7. In the study of Fernandez *et al.* (2008) stress treatment of growing *Lactobacillus bulgaricus* ATCC11842 cells (pH 3.8, 35 min at 42 °C) clearly resulted in induction of *clpL* while transcriptional analysis revealed no induction (Fernandez *et al.*, 2008). To our knowledge no other studies involving the measurement of the expression of *clpL* have been performed in conjunction with acid stress treatments in lactic acid bacteria. It is possible that the moderate acid treatment conditions used in the present study were not harsh enough to induce a measurable expression of *clpL1* or *clpL2* in *L. rhamnosus* E800. Also, the stress treatments procedure used in previous studies and the physiological state of the cells differ from our study, which possibly explain the difference of results.

Although culture-based analysis indicated that the viability of the freeze-dried samples was high, mRNA from *L. rhamnosus* E800 powders could not be amplified with either *clpL1* or *clpL2* primers. This probably reflects the difficulty in isolating RNA from freeze-dried prokaryotic cell material. In addition, the carrier (polydextrose) used in the freeze-drying might have interfered with the mRNA isolation or PCR reaction inhibiting the amplification. To our knowledge this was the first attempt to isolate mRNA from freeze-dried bacterial cells. Previously RT-qPCR has been successfully applied in the gene expression analysis of dried yeast preparations (Vaudano *et al.*, 2009).

In conclusion, our results suggest that the induction of *clpL* genes in *L. rhamnosus* E800 is detectable with RT-qPCR in the case where heat stress is fairly extreme. Thus, although an induction of *clpL1* was seen during heat stress conditions applied in this study, the expression of this gene can not be considered a good candidate as a marker to study the quality of *L. rhamnosus* products. Furthermore, mRNA-based RT-qPCR analysis is a laborious and time consuming technique for routine use, and the fact that it is difficult to

obtain mRNA from freeze-dried bacterial powders sets an additional limitation for the applicability of this technique in quality control of probiotic products.

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Dietary effects on human fecal microbiota

The establishment of microbial populations in the gastrointestinal tract is a complex process, involving microbial and host interactions eventually resulting in a dense and stable population. Recently, the identification and quantification of microbial species from fecal samples has become more accurate with the use of 16S rRNA gene-based methods.

Different storage conditions and DNA-extraction protocols affect the numbers of the most abundant bacterial groups in fecal samples as measured with qPCR in this study. In particular, rigorous mechanical lysis of human fecal samples led to the detection of higher bacterial numbers than enzymatic DNA-extraction, particularly in the case of Actinobacteria.

The composition of the human intestinal microbiota is influenced by host factors such as age, genetics, secretory products such as gastric acid and bile, peristalsis and gastrointestinal transit time. On the other hand, environmental factors with impact on the host during the lifespan, such as diet, continuously modulate this microbial community.

The present study confirms that the dietary and energetic intake modulates bacterial groups within the fecal microbiota. In particular, different types of dietary fat have distinct effects on the fecal microbiota composition, suggesting that a balanced diet with regard to fat consumption is critical not only for the host's health but also for the intestinal microbiota. In addition, dietary intake within monozygotic twin pairs may be more influential than body fat levels in determining the fecal microbiota. Moreover, during the 12 month weight loss intervention of this study, the change in fecal microbial numbers correlated with the energy intake and not with the change in weight. Therefore, studies on the relationship between gut microbiota and health of the host should not rely only on BMI values but should also consider other variables such as diet composition.

The potential of utilising the information on expression levels of selected stress genes in assessing the quality of probiotic products was also evaluated in this study. The induction of clpL genes in *Lactobacillus rhamnosus* VTT-E97800 appears to be detectable with reverse transcription-qPCR only in cases when the heat or acid stress is extreme. Therefore, although an induction of clpL1 was observed during the heat stress conditions applied, the expression of this gene cannot be considered to be a good candidate as a marker to study the quality of *L. rhamnosus* roducts.

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