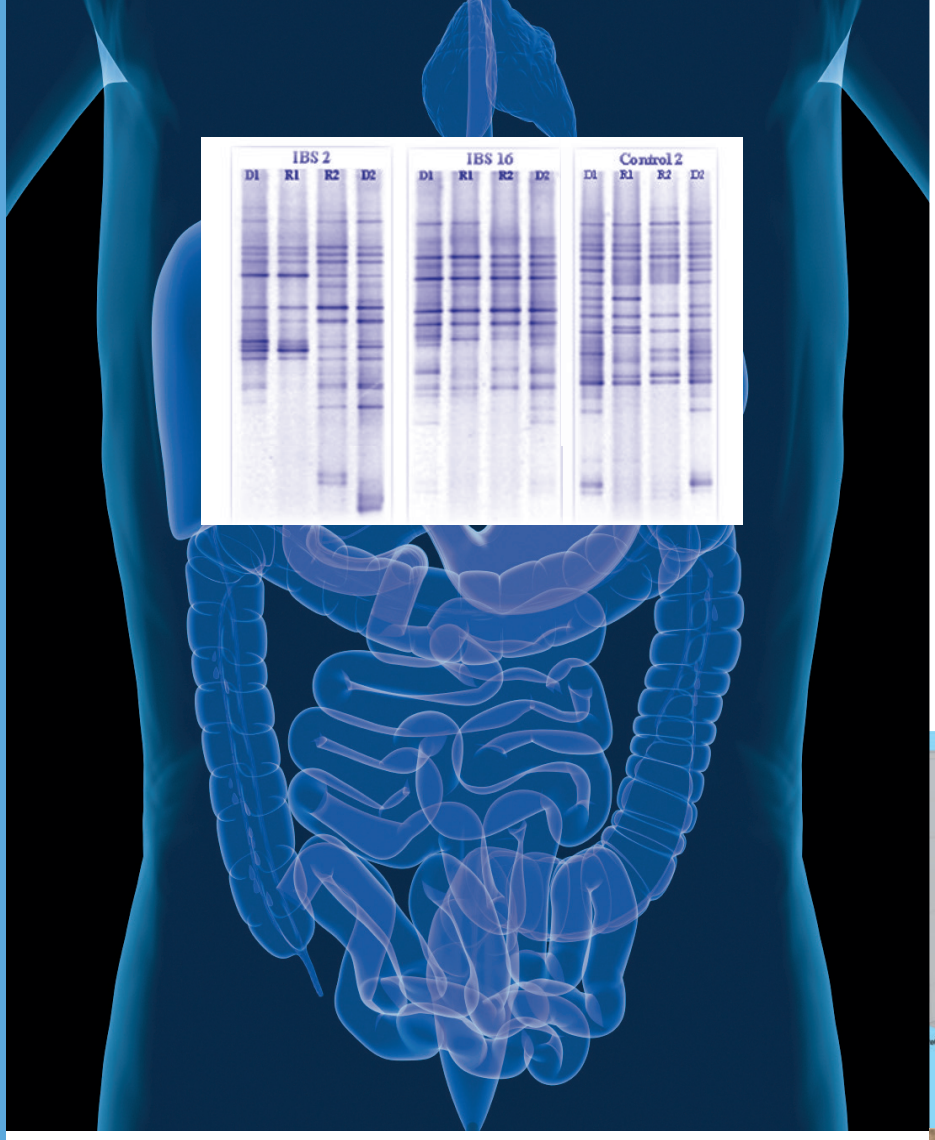

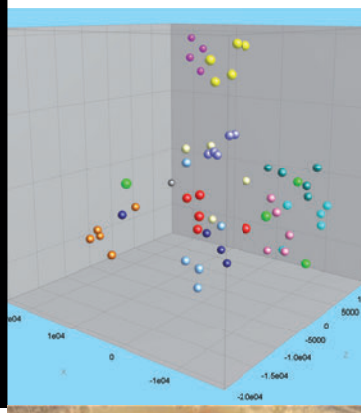

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Dissertation
26

Characterization of the human predominant fecal microbiota

With special focus on the Clostridial clusters IV and XIVa

Johanna Maukonen

VTT

VTT SCIENCE 26

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Johanna Maukonen

Doctoral dissertation for the degree of Doctor of Science in Technology to be presented with due permission of the School of Chemical Technology for public examination and debate in Auditorium KE2 (Komppa Auditorium) at the Aalto University School of Chemical Technology (Espoo, Finland) on the 7th of December, 2012, at 12 noon.

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Characterization of the human predominant fecal microbiota
With special focus on the Clostridial clusters IV and XIVa

Ihmisen vallitsevan ulostemikrobiston karakterisointi. Painopiste erityisesti klostridien fylogeneettisissä ryhmissä IV ja XIVa. **Johanna Maukonen.**
Espoo 2012. VTT Science 26. 161 p. + app. 62 p.

Abstract

The human gut microbiota is considered to be a complex fermentor with a metabolic potential rivaling that of the liver. In addition to its primary function in digestion, it affects the human host in numerous ways: maturation and modulation of the immune system, production of short-chain fatty acids and gases, transformation of bile acids, formation of vitamins, and also potential formation of mutagenic, toxic, and carcinogenic substances. Commensal bacteria are able to modulate the expression of host genes that regulate diverse and fundamental physiological functions. Thus the indigenous microbial community has an important influence on host physiological, nutritional and immunological processes. The primary aim of this study was to characterize human predominant fecal microbiota with a special focus on Clostridial clusters XIV (Lachnospiraceae, *Eubacterium rectale – Blautia coccoides* group) and IV (Ruminococcaceae, *Clostridium leptum* group). The specific aims were: 1) To develop molecular methods for characterization of the human predominant fecal microbiota; 2) To assess the specificity, practicality, and usability of the developed methods for human fecal samples in healthy adults, elderly people, and people having IBS; 3) To assess possible confounding factors in the analysis of human fecal samples.

Molecular tools were developed for rapid, sensitive, and highly specific characterization of the human predominant fecal and salivary microbiota. DNA- and rRNA-based denaturing gradient gel electrophoresis methods (DGGE) were developed for *Eubacterium rectale – Blautia coccoides* group (Erec), rRNA-based DGGE method for predominant bacteria, and DNA-based DGGE methods for *Clostridium leptum* group (Clept) and *Bacteroides* spp. In addition, quantitative real-time PCR (qPCR) methods targeting predominant bacteria, Erec-group, Clept-group, *Bacteroides* spp., bifidobacteria, and *Atopobium* group were developed.

Predominant bacterial, Erec-group, Clept-group, and *Bacteroides* spp. populations of healthy adults were temporally rather stable, showing intra-individual diversity and inter-individual variability. The rRNA-based profiles showed more temporal instability than DNA-based profiles. The enumerated clostridial groups (Erec, Clept, *C. lituseburense,* and *C. histolyticum*) represented the dominant fecal microbiota of most of the studied subjects, comprising altogether 29–87% of the total bacteria. Erec-group was the dominant group, accounting on average for 43% of total bacteria in control subjects, and 30% and 50% in irritable bowel syndrome (IBS) subjects affected by constipation and diarrhea, respectively. The proportion of Erec-group was significantly lower in the constipation type IBS subjects than in

the healthy controls. The rRNA-based DGGE-profiles of the fecal microbiota of the IBS subjects further indicated higher temporal instability of their microbiota composition as compared to the control subjects. Our observations indicated that in addition to temporal instability of the active predominant fecal bacterial population, clostridial microbiota may be involved in IBS.

Differences in the predominant fecal microbiota between elderly people and younger adults were also assessed. Temporal stabilities of the studied bacterial populations (predominant bacteria, Erec-group, bifidobacteria, and lactobacilli) were similar in both age groups. However, the diversity of predominant bacteria and Erec-group bacteria was significantly higher in elderly subjects as compared to younger adults. Consumption of probiotic yoghurt containing galacto-oligosaccharide (GOS) for three weeks did not significantly affect the diversity or temporal stability of the studied bacterial groups. However, the composite data set containing all DGGE analyses of the study showed that the microbial communities from the GOS-supplemented subjects were more similar to each other than those of the control subjects. Therefore, the GOS-yoghurt may have a stabilizing effect on the predominant fecal microbiota of elderly people.

Thereafter, the similarity of the salivary and fecal microbiota was studied to assess whether the upper gastrointestinal tract microbiota influence the results obtained with DNA-based methods from feces. The predominant bacteria, bifidobacteria, and Erec-group bacteria of the oral cavity and feces were generally stable during probiotic consumption, showing more diversity in feces than in saliva and different species compositions for the two sampling sites. Lactobacilli, however, showed temporal instability in both feces and saliva. Furthermore, fecal and salivary samples contained identical indigenous *Lactobacillus* genotypes (*L. rhamnosus*, *L. gasseri*, *L. paracasei*, *L. plantarum* group, and a *Lactobacillus* sp.) in most subjects.

The effects of storage conditions and DNA-extraction protocols of fecal samples on the results were also evaluated. The DNA-extraction did not affect the diversity, composition, or quantity of *Bacteroides* spp., whereas after one week's storage at -20^{o}C the numbers of *Bacteroides* spp. were 1.6–2.5 log-units lower ($p<0.05$). Furthermore, the numbers of predominant bacteria, Erec-group, Clept-group, bifidobacteria, and *Atopobium*-group were 0.5–4 log-units higher ($p<0.05$) after mechanical DNA-extraction than after enzymatic DNA-extraction as detected with qPCR, regardless of the storage. The bacterial composition of Erec-group differed significantly depending on the DNA-extraction protocol; 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 the results, rigorous mechanical lysis leads to the detection of higher bacterial numbers and diversity from human fecal samples than enzymatic DNA-extraction. Since 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.

Keywords human fecal microbiota, human salivary microbiota, DGGE, qPCR, IBS, elderly, GOS, DNA-extraction, Erec, Clept, *Bacteroides* spp., bifidobacteria, lactobacill

Ihmisen vallitsevan ulostemikrobiston karakterisointi
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Tiivistelmä

Suolistomikrobiston ensisijainen tehtävä on auttaa ruoansulatuksessa; paksusuolen mikrobit käyttävät hyödykseen useita ravintoaineita, jotka eivät imeydy ohutsuolessa. Ihminen puolestaan hyödyntää mikrobien aineenvaihduntatuotteita. Suolistomikrobisto vaikuttaa ihmiseen monella muullakin tavoin: se edesauttaa immuunijärjestelmän kehittymistä, tuottaa kaasuja sekä lyhytketjuisia rasvahappoja, muuttaa sappihappojen muotoa, tuottaa vitamiineja ja muodostaa mahdollisesti myös mutageenisiä, toksisia sekä karsinogeenisiä yhdisteitä. Niinpä ihmisen suolistomikrobistolla on merkittävä vaikutus ihmisen fysiologisiin, ravitsemuksellisiin ja immunologisiin toimintoihin. Tämän tutkimuksen tavoitteena oli kehittää menetelmiä ihmisen suolistomikrobiston tärkeimpien bakteeriryhmien karakterisointiin ja kvantitointiin ja todentaa kehitettyjen menetelmien soveltuvuus ihmisen uloste- ja sylkinäytteiden analysointiin.

Tässä työssä kehitettiin spesifiset ja herkät rRNA-pohjaiset PCR – denaturoiva gradientti geeli elektroforeesi (DGGE) -menetelmät vallitsevan bakteeriston ja *Eubacterium rectale – Blautia coccoides* -klostridiryhmän (Erec) karakterisointiin sekä DNA-pohjaiset PCR-DGGE-menetelmät *Clostridium leptum* -klostridiryhmän (Clept), sekä *Bacteroides*-suvun karakterisointiin. Lisäksi kehitettiin reaaliaikaiset PCR-menetelmät (qPCR) vallitsevan bakteeriston, Erec-ryhmän, Clept-ryhmän, *Bacteroides*-suvun, bifidobakteerien, sekä *Atopobium*-ryhmän kvantitointiin.

Terveiden aikuisten vallitseva bakteeriston, Erec-klostridiryhmän, Clept-klostridiryhmän ja *Bacteroides*-suvun bakteeripopulaatiot olivat ajallisesti melko stabiileja. Bakteeripopulaatioprofiilit olivat monimuotoisia ja kullekin ihmiselle ominaisia. rRNA:han pohjautuvat bakteeriprofiilit olivat sekä terveillä aikuisilla että ärtyneen suolen oireyhtymästä (IBS) kärsivillä aikuisilla ajallisesti epästabiilimpia kuin DNA-pohjaiset bakteeriprofiilit. Lisäksi IBS-potilaiden rRNA-pohjaiset vallitsevan bakteeriston profiilit olivat ajallisesti epästabiilimpia kuin terveiden aikuisten ($p < 0.05$). Tutkittujen klostridiryhmien (Erec, Clept, *C. lituseburense* -ryhmä sekä *C. histolyticum* -ryhmä) bakteerit muodostivat valtaosan (29–87 %) ulostemikrobistosta. Erec-ryhmän klostridit muodostivat keskimäärin 43 % terveiden aikuisten ulostemikrobistosta, kun taas IBS-potilailla Erec-ryhmän klostridit muodostivat 30 % ($p < 0.05$; ummetustyyppi) tai 50 % (ripulityyppi) ulostemikrobistosta.

Aikuisten ja vanhempien ihmisten (yli 60 v.) välillä ei ollut eroja tutkittujen bakteeriryhmien ajallisessa stabiilisuudessa (vallitseva bakteeristo, Erec-ryhmän klostridit, bifidobakteerit ja laktobasillit). Vanhempien ihmisten vallitseva bakteeristo sekä Erec-klostridiryhmä olivat kuitenkin monimuotoisempia kuin nuorempien

aikuisten. Galakto-oligosakkarideja (GOS) sisältävällä probioottisella jogurtilla ei ollut vaikutusta vanhempien ihmisten ulostmikrobiston monimuotoisuuteen eikä ajalliseen stabiilisuuteen. GOS-jogurtti kuitenkin muokkasi vanhempien ihmisten ulostemikrobistoa siten, että ulostemikrobistot muistuttivat enemmän toisiaan GOS-jogurtti jakson jälkeen kuin lumejakson jälkeen.

Koska DNA-pohjaisilla menetelmillä voitaisiin periaatteessa detektoida ulosteesta myös sylkibakteereita, tutkimuksesssa selvitettiin myös, löytyykö syljestä samoja bakteereita kuin ulosteesta. Sekä syljen että ulosteen vallitseva bakteeristo, Erec-ryhmän klostridit, bifidobakteeri- sekä laktobasillipopulaatiot pysyivät melko stabiileina probiootti-syöttöjakson aikana. Ulostenäytteiden bakteeripopulaatiot olivat monimuotoisempia kuin syljen bakteeripopulaatiot. Lisäksi näiden bakteerisuvut/lajit olivat pääasiallisesti erilaisia. Ainoan selkeän poikkeuksen muodostivat laktobasillit; useimpien koehenkilöiden syljestä ja ulosteesta löytyi samoja laktobasilli-lajeja ja toisinaan jopa kantoja (esim. *L. rhamnosus*, *L. gasseri*, *L. paracasei*, *L. plantarum*-ryhmä ja *Lactobacillus* sp.).

Viimeisessä osatyössä tutkittiin ulostenäytteiden säilytyslämpötilojen ja eri DNA-eristysmenetelmien vaikutusta näytteistä saataviin tuloksiin. Näytteiden säilytys -20 ^{o}C:ssa viikon ajan alensi *Bacteroides*-suvun bakteerien lukumäärää 1,6–2,5 log-yksikön verran ($p < 0.05$), kun taas eri DNA-eristysmenetelmillä ei ollut vaikutusta *Bacteroides*-suvun bakteerien määrään tai populaation monimuotoisuuteen tai koostumukseen. Säilytyslämpötila ei vaikuttanut merkitsevästi muiden tutkittujen bakteeriryhmien määriin eikä myöskään populaatioiden monimuotoisuuteen tai koostumukseen. Käytetyllä DNA-eristysmenetelmällä sen sijaan oli merkittävä vaikutus: Vallitseva bakteeriston, Erec-ryhmän klostridien, Clept-ryhmän klostridien, bifidobakteerien ja *Atopobium*-ryhmän bakteerien määrät olivat 0,5–4 log-yksikköä korkeammat ($p < 0.05$) mekaanista DNA-eristystä käytettäessä kuin entsymaattista DNA-eristystä käytettäessä. Lisäksi käytetyllä DNA-eristysmenetelmällä oli suuri vaikutus Erec-ryhmän bakteerikoostumukseen: Mekaanisen DNA-eristyksen jälkeen yleisimmät detektoidut suvut olivat *Blautia* (30 % klooneista), *Coprococcus* (13 %) ja *Dorea* (10 %), kun taas entsymaattisen DNA-eristyksen jälkeen yleisimmät suvut olivat *Roseburia* (39 %) ja *Coprococcus* (10 %). Käytetyllä DNA-eristysmenetelmällä on saatujen tulosten perusteella huomattava merkitys etenkin klostridi- sekä aktinobakteeripopulaatioden detektointiin ja kvantitointiin. Eri DNA-eristysmenetelmien käyttö saattaakin osittain selittää viime aikoina saatuja ristiriitaisia tuloksia liittyen esim. suolistomikrobiston rooliin lihavuudessa ja bifidobakteerien määrään vauvojen suolistossa.

Avainsanat human fecal microbiota, human salivary microbiota, DGGE, qPCR, IBS, elderly, GOS, DNA-extraction, Erec, Clept, *Bacteroides* spp., bifidobacteria, lactobacilli

Preface

The studies presented in this thesis were carried out at VTT Technical Research Centre of Finland during the years 2002–2012. The financial support of European Commission [ARTRADI (QLK2-CT-2002-00843), TORNADO (FP7-KBBE-222720), and ETHERPATHS (FP7-KBBE-222639)], Academy of Finland (SA-klostridi; project 209758), the Finnish Funding Agency for Technology and Innovation (IBSPROB; Tekes grant 954/401/00), and VTT is greatly appreciated.

I am grateful to Vice Presidents R&D, Bio and Process Technology, Prof. Juha Ahvenainen (–2007) and Prof. Anu Kaukovirta-Norja (2007–), and Technology Manager Dr. Richard Fagerström, as well as to my team leader Doc. Maria Saarela for providing excellent working facilities and for the possibility to finalize this thesis.

I express my sincere gratitude to the pre-examiners Prof. Michael Blaut and Prof. Atte von Wright for their detailed interest in my thesis, although the manuscript was rather long and the schedule rather tight. Their suggestions clearly improved the manuscript. I warmly thank Prof. Katrina Nordström for her cooperation during the preparation of this thesis.

I am deeply grateful to my supervisor Doc. Maria Saarela for introducing me to the captivating world of human oro-gastrointestinal microbiota. I also warmly thank her for continuous interest in my studies, all the philosophical discussions, and for being available whenever I have needed help (regardless of her semi-nomadic job description). I am also grateful to Dr. Jaana Mättö for fruitful discussions and invaluable advice related to the method developments, while you were still working at VTT.

I warmly thank all my co-authors, Dr. Jaana Mättö, Prof. Tiina Mattila-Sandholm, Doc. Reetta Satokari, Prof. Hans Söderlund, Doc. Maija-Liisa Suihko, Dr. Kajsa Kajander, and MSc Catarina Simões for pleasant cooperation during the projects and while preparing the manuscripts. Michael Bailey is thanked for his fast and excellent language editing. I also thank Prof. Sirkka Asikainen for providing three bacterial strains for method validation (*Fusobacterium nucleatum*, *Prevotella melaninogenica*, and *Veillonella parvula*).

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I sincerely thank all my colleagues at VTT for cooperation during the past 17 years. Although only a few of the past studies are included in this thesis, all the projects and cooperation during my years at VTT are greatly acknowledged. The different types of projects, protocols, and people have taught me a lot. Especially I want to thank my present and past colleagues from the 2nd and 3rd floor microbiology laboratories: Anne, Annukka, Arja, Auli, Catarina, Eija, Erna, Gun, Hanna, Helena, Kari, Kirsi, Maija-Liisa, Maisa, Mari, Maria, Merja, Mia, Niina, Outi, Reetta, Riikka, Satu, Tarja N, Tiina H., Tiina K., Tuija and all the others for creating such a tremendous working atmosphere. There has never been a dull (or quiet…) day!

Last but not least, I heartily thank my parents and brother for their love and support throughout my life. I also warmly thank my mother Anna-Liisa for countless hours of baby-sitting (which have enabled the completion of this thesis), brother Juha for mathematical advice (despite my stupid questions posed at odd hours), and mother Anna-Liisa & father Antti for financing my foreign exchange year many, many years ago; the language skills obtained during that year have smoothed my way in the scientific – English-speaking – world. My special thanks are due to all my dear friends, especially Elina, Jyri, Kirsi K., Kirsi T. & Satu, who have shared the joys and sorrows of everyday life. Finally, I express my heartfelt thanks to my husband Marko and to my children Juho & Anni for their love and patience during these years. They have ensured that I have not worked 24/7 (although I have occasionally tried), by reminding me what is most important in life.

Espoo, October 2012

Johanna Maukonen

Academic dissertation

Custos	Professor Katrina Nordström School of Chemical Technology Aalto University Espoo, Finland
Supervisor	Docent Maria Saarela VTT Technical Research Centre of Finland Espoo, Finland
Reviewers	Professor Michael Blaut PhD German Institute of Human Nutrition Nuthetal, Germany Professor Atte von Wright Institute of Public Health and Clinical Nutrition University of Eastern Finland Kuopio, Finland
Opponent	Professor Airi Palva Faculty of Veterinary Medicine University of Helsinki Helsinki, Finland

List of publications

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data is presented.

I. Maukonen J, Satokari R, Mättö J, Söderlund H, Mattila-Sandholm T & Saarela M (2006). Prevalence and temporal stability of selected clostridial groups in irritable bowel syndrome in relation to predominant faecal bacteria. *Journal of Medical Microbiology,* 55, 625–633.

II. Maukonen J, Mättö J, Satokari R, Söderlund H, Mattila-Sandholm T & Saarela M (2006). PCR-DGGE and RT-PCR-DGGE show diversity and short-term temporal stability in the *Clostridium coccoides – Eubacterium rectale* group in the human intestinal microbiota. *FEMS Microbiology Ecology*, 58, 517–528.

III. Maukonen J, Mättö J, Kajander K, Mattila-Sandholm T & Saarela M (2008). Diversity and temporal stability of fecal bacterial populations in elderly subjects consuming galacto-oligosaccharide containing probiotic yoghurt. *International Dairy Journal*, 18, 386–395.

IV. Maukonen J, Mättö J, Suihko M-L & Saarela M (2008). Intra-individual diversity and similarity of salivary and faecal microbiota. *Journal of Medical Microbiology*, 57, 1560–1568.

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

Author's contributions

I Johanna Maukonen had the main responsibility for preparing and writing the article, and is the corresponding author. She planned the study together with the co-authors. She designed the experiments and did the experimental work together with Reetta Satokari, and was mainly responsible for the interpretation of the results. Most of the experimental work related to transcriptional profiling with the aid of affinity capture was performed by Reetta Satokari, but Johanna Maukonen also participated with a smaller impact.

II Johanna Maukonen had the main responsibility for preparing and writing the article, and is the corresponding author. She was mainly responsible for the planning of the study, design of the experiments, experimental work, and interpretation of the results.

III Johanna Maukonen had the main responsibility for preparing and writing the article, and is the corresponding author. She planned the study together with the co-authors. She was mainly responsible for the design of the experiments and interpretation of the results. She was partly responsible for the experimental work, and supervised the rest.

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V Johanna Maukonen had the main responsibility for preparing and writing the article, and is the corresponding author. She was mainly responsible for the planning of the study, design of the experiments, and interpretation of the results. She was partly responsible for the experimental work, except for the qPCR studies, which were performed by Catarina Simões.

Contents

List of abbreviations

ATCC	American Type Culture Collection
AFM	atomic force microscope
ARDRA	amplified rDNA restriction analysis
CD	Crohn's disease
CFU	colony forming unit
Chis	*Clostridium histolyticum* group of clostridia (Clostridial cluster II), however, the Chis-probe targets both Clostridial clusters I and II
Clept	*Clostridium leptum* group of clostridia (Clostridial cluster IV; Ruminococcaceae)
Clit	*Clostridium lituseburense* group of clostridia (Clostridial cluster XI)
CH_4	methane
CO_2	carbon dioxide
CRC	colorectal cancer
C-section	Cesarean section
CSLM	confocal scanning laser microscopy
DAPI	4',6-diamidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)
Erec	*Eubacterium rectale – Blautia coccoides* group of clostridia (Clostridial cluster XIV; Lachnospiraceae)
FISH	fluorescent *in situ* hybridization
FOS	fructo-oligosaccharide
G+C	guanine + cytosine
GI	gastrointestinal
GIT	gastrointestinal tract
GOS	galacto-oligosaccharide
H_2	hydrogen
H_2S	hydrogen sulfide
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome

LAB	lactic acid bacteria
LPS	lipopolysaccharides
MA	methanogenic archaea
MIC	minimal inhibitory concentration
MRSA	de Man – Rogosa – Sharpe agar
mRNA	messenger ribonucleic acid
MZ	monozygotic
NGS	next-generation sequencing
OUT	operational taxonomic unit
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PYG	peptone-glucose-yeast (medium)
qPCR	quantitative (real-time) PCR
RAPD	randomly amplified polymorphic deoxyribonucleic acid
rDNA	ribosomal deoxyribonucleic acid
mRNA	messenger ribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SEM	scanning electron microscope
SRB	sulfate reducing bacteria
SSCP	single-stranded conformation polymorphism
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TEM	Transmission electron microscope
TGGE	temperature gradient gel electrophoresis
TGY	trypticase- glucose-yeast extract
TRAC	transcriptional profiling with the aid of affinity capture
T-RFLP	terminal restriction fragment length polymorphism
UC	ulcerative colitis

1. Introduction

The human oro-gastrointestinal tract (oro-GIT) is a complex system, consisting of oral cavity, pharynx, esophagus, stomach, small intestine, large intestine, rectum, and anus (Figure 1), which together with the accessory digestive organs (e.g. teeth, tongue, salivary glands, liver, gallbladder, and pancreas) constitute the digestive system. The function of the digestive system is to break down dietary constituents into small molecules and then absorb these for subsequent distribution throughout the body[1].

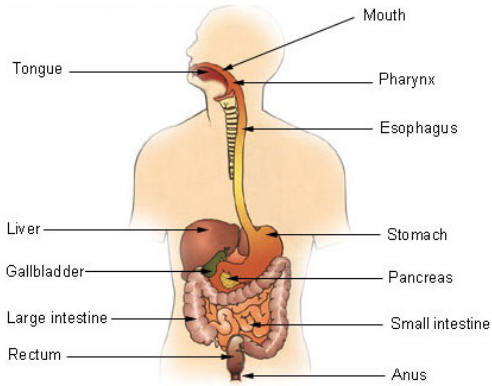

Figure 1. The main anatomical features of the human oro-gastrointestinal tract (From: SEER Training Modules, Digestive system. U.S. National Institutes of Health, National Cancer Institute. May 11th, 2010. (<*http://training.seer.cancer.gov/anatomy/digestive/regions/*>)

In addition to digestion and carbohydrate metabolism[2], the oro-GIT microbiota affects the human host in numerous ways including maturation and modulation of the immune system[3], production of short-chain fatty acids[4-5] and gases such as H_2, CO_2, and CH_4[6], transformation of bile acids[7-8], formation of vitamins[4,9] and biodegradation of xenobiotics[4], and is also involved in the potential formation of mutagenic, toxic[10], and carcinogenic substances[11]. In addition, the indigenous microbial community has an important influence on host physiological, nutritional and immunological processes, and commensal bacteria are able to modulate the expression of host genes that regulate diverse and fundamental physiological functions[12].

In an adult human, resident bacteria outnumber human cells by a factor of ten – each adult harbors on average 10^{13} mammalian cells and 10^{14} microbial cells[13]. Most of these

microbes, typically 10^{11}–10^{12} microbes/g, are located in the feces and large intestine[14-16], which is considered to be a complex fermentor with a metabolic potential rivaling that of the liver[17]. The environmental determinants – namely temperature, pH, redox potential, atmospheric composition, water activity, salinity, and light – within each region of the human oro-GIT are very different, and therefore each region has its own distinctive microbiota[1].

The aim of this PhD thesis was to study the commensal fecal microbiota, and therefore pathogenic bacteria are not discussed in detail in the following sections.

1.1 The microbiota of the human oro-gastrointestinal tract

1.1.1 Oral cavity

The oral cavity is formed by the cheeks, the hard and soft palates, and the tongue (Figure 2), and it is connected to the pharynx. The total surface area of the oral cavity, including teeth, is approximately 200 cm^2, of which tooth surfaces account for ~20%. The hard palate separates the oral and nasal cavities, and the soft palate the oropharynx and nasopharynx[1].

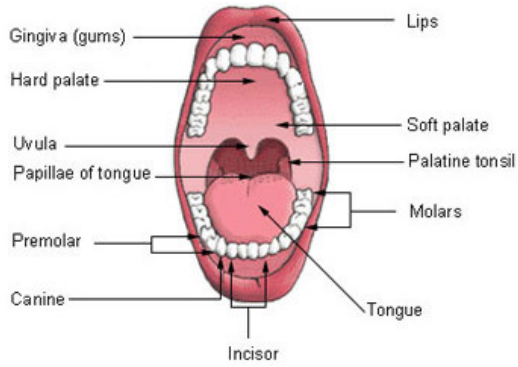

Figure 2. The main anatomical features of the human oral cavity (From: SEER Training Modules, Digestive system. U.S. National Institutes of Health, National Cancer Institute. May 12th, 2010. (<*http://training.seer.cancer.gov/anatomy/digestive/regions/mouth.html*>)

Each different micro-environment (cheeks, palate, tongue, tooth surfaces, gingival areas, and saliva) contains its own microbiota[18-19], partly due to the different environmental conditions. The temperature of the oral cavity varies from site to site, but remains mainly within the range of 33–37°C. However, when cold or hot drinks and food are consumed, the temperature variations may be considerably larger. The pH of saliva, tongue and mucosal surfaces is usually approximately neutral, whereas the pH of gingival crevices is alkaline and that of fissures and approximal oral regions tends to be neutral/acidic. In addition, the redox potential of most of the oral sites is positive (E^0 = 74-273 mV). However, the redox potential of gingival crevices is negative (E^0 = -73 mV)[1], thereby offering a suitable niche for obligate anaerobes.

Communities in the oral cavity are polymicrobial and exist primarily as biofilms on the surface of the teeth, gums, and tongue. Over 600 prevalent oral bacterial taxa are recog-

nized, of which approximately 50% have not yet been cultured[20-21]. The number of predominant species – including all oral sites – may vary individually between 34 and 72 as detected with Sanger sequencing[18] and between 88 and 104 as detected with pyrosequencing based on ~240 nt long sequences[19]). The predominant taxa that have been detected in various sites of the oral cavity with molecular techniques include: phyla Firmicutes (e.g. *Streptococcus, Gemella, Eubacterium, Selemonas*, *Veillonella,* and *Granulicatella* species), Actinobacteria (e.g. *Actinomyces, Atopobium, Rothia, Corynebacterium,* and related species), Fusobacteria (e.g. *Fusobacterium* and *Leptotrichia* species), Proteobacteria (e.g. *Neisseria, Eikenella, Kingella, Campylobacter,* and related species), Bacteroidetes (e.g. *Prevotella, Porphyromonas, Parabacteroides,* and *Capnocytophaga* species), and TM7 (no culturable representatives). In addition, smaller populations of the phyla Cyanobacteria, Spirochaetes, Chlamydiae, Chloroflexi, Euryarchaeota, SR1, Synergistetes, and Tenericutes have been detected in different oral sites[18-19,21-25]. In the oral cavity, indigenous lactobacilli and bifidobacteria do not have the same positive image as in the gut, since they have been associated with oral diseases. In addition, they can be detected but their occurrence is reversed as compared to feces: lactobacilli are a common finding in the oral cavity whereas bifidobacteria are less frequently detected. *Lactobacillus* species found in oral samples include *L. casei group*, *L. acidophilus* group, *L. plantarum*, *L. fermentum*, and *L. salivarius*[26-27]. The oral cavity harbors only one known indigenous *Bifidobacterium* species, *B. dentium*[28]. Other oral *Bifidobacterium*-like organisms currently belong to the genera *Scardovia* and *Parascardovia* (*Scardovia inopinata* and *Parascardovia denticolens*[29]. Altogether, culturable and not yet culturable species of *Gemella, Granulicatella, Streptococcus*, and *Veillonella* have commonly been found in most of the oral sites[18-19]. Moreover, 30 significant health-associated species – i.e. species that are more prevalent in healthy than in diseased mouth; e.g. *Streptococcus sanguinis, Kingella oralis,* and *Streptococcus intermedius* – have been found at healthy sites (i.e. no caries, gingivitis, or periodontitis) in permanent teeth[30-32].

The number of bacteria in the oral cavity is approximately 10^{11} per gram (wet weight) of dental plaque and 10^{8}–10^{9} per ml of saliva (culturable bacteria[33-34]). Salivary microbiota reflects a mixture of bacteria washed off from the various surfaces of the oral cavity, especially from the tongue[34]. The whole saliva also contains gingival crevicular fluid (GCF), mucosal transudations, expectorated bronchial and nasal secretions, serum and blood derivatives from oral wounds, desquamated epithelial cells, other cellular components, and food debris[35]. Since saliva is easily available, it has been a widely used sample material in oral microbiota studies. The bacterial microbiota of saliva is dominated by the genera *Streptococcus*, *Prevotella*, *Veillonella*, *Neisseria*, *Haemophilus*, *Rothia*, *Porphyromonas*, and *Fusobacterium*, which account for over 70% of the microbiota[18-19,24-25]. In addition, high microbial diversity within and between individuals has been found, but only minimal grouping based on the host's geographical location[24]. In a study of Yang *et al.* only a minimal core salivary microbiome was found, comprising of 0.53% of the detected bacteria[25]. Instead Zaura *et al.* found a clear core microbiome, which comprised of 47% of the detected bacteria[19]. This discrepancy is partly due to the stringent read-selection of Zaura *et al.*[19], which resulted in exclusion of sequences that were found less than five times. In addition, in the study of Zaura *et al.* only three study subjects were included, whereas in the study of Yang *et al.* 26 study subjects were included. Therefore, it is likely that the conclusions of Yang *et al.*[25] are more accurate.

1.1.2 Pharynx and esophagus

The pharynx (Figure 1; Section 1) is a tube that extends from the internal nares down to the larynx and consists of three main regions: the nasopharynx, the oropharynx, and the laryngopharynx. The pharynx acts as a passageway for food, drink, and air[1]. The oropharynx is constantly exposed to both ingested and inhaled microbes, those contained in saliva and those cleared by mucociliary mechanisms from both the upper and lower respiratory tracts. Members of nine microbial phyla (Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria, Actinobacteria, TM7 and SR1), represented by 71 taxa have been found in the oropharynx. The majority of the bacteria belonged to Streptococcaceae, as well as to Lachnospiraceae and Acidaminococcaceae[36].

The esophagus (Figure 1; Section 1) connects the laryngopharynx to the stomach, and its function is to transport food between these regions. The esophagus is an aerobic region. Food entering the esophagus moves towards the stomach by peristalsis, and movement of the food bolus is aided by the presence of saliva and mucus. The temperature in esophagus is 37^{o}C and pH of esophagus mucus layer is ~6.8[1]. Overall, representatives of nine microbial phyla, represented by 166 species have been found in the esophagus. Based on these results, the distal esophagus may harbor over 200 species, as predicted by the Chao1 richness estimator[37]. The predominant genus in the esophagus of healthy individuals is *Streptococcus* (>78% of clones), and in particular the mitis group species *S. mitis* and *S. pseudopneumoniae*[25,38-40]. Moreover, *S. mitis* was the only taxon that was found in all samples in the study of Yang *et al.*[37]. Gram-negative bacteria (e.g. *Prevotella, Veillonella* etc.) comprised on average 15% of the clones. Moreover, the aerobic conditions of the site were reflected in the population structure, since only 16% of the clones originated from anaerobic or microaerophilic bacteria[37]. Overall, the bacterial population in the normal distal esophagus is similar to that of the oropharynx[22-23]. The majority of clones, especially those that share significant homology only with uncultivated 16S rRNA gene clones, are mostly related to oral bacteria, suggesting that transient bacteria originating from the oral cavity predominated in the specimens[39].

The microbiota of the throat has been found to be similar to that of the distal esophagus; eight genera (*Streptococcus, Prevotella, Actinomyces, Gemella, Rothia, Granulicatella, Haemophilus,* and *Veillonella*) were found in all the samples, constituting 75% of the total sequences. Genus *Streptococcus* was the dominant genus followed by *Prevotella*. A differentiating genus between the throat and esophagus microbiota was *Veillonella*, representing 14% of the esophagus sequences but only 0.4% of the throat reads[39-41].

1.1.3 Stomach

The stomach is a J-shaped structure linking the esophagus to the duodenum (Figure 3) and has a total capacity of approximately 1500 ml[1].

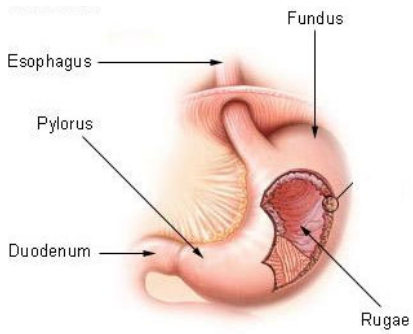

Figure 3. The main anatomical features of the human stomach (From: SEER Training Modules, Digestive system. U.S. National Institutes of Health, National Cancer Institute. May 12th, 2010. (<*http://training.seer.cancer.gov/anatomy/digestive/regions/stomach.html*>)

The temperature of the gastric fluid is usually 37°C, but may vary due to intake of hot and cold foods and drinks. The stomach is a partly aerobic environment, having a partial pressure of oxygen which corresponds to 29% of the oxygen content of air. The median 24 h intragastric pH is 1.4. However, several factors influence the individual pH – namely age, diet, medication, and whether or not food/drinks have recently been ingested. Especially food has a strong buffering action, causing a pH variation of 1 to 5, of which the higher pH corresponds to meal times[1]. In addition, proton pump inhibitors, which are commonly used for the treatment of gastroesophageal reflux disease and acid-related diseases, increases the intragastric pH[42].

Traditionally, characterization of the gastric microbiota has relied on cultivation of gastric juice or mucosal biopsies. In these studies, several members of the phyla Firmicutes, Proteobacteria, Actinobacteria, and Fusobacteria, as well as yeasts in relatively low abundance have been identified[13,43]. However, in studies using a molecular approach[41, 44-45], more diverse communities have been identified, in which the majority of sequences have belonged to the phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Fusobacteria. In addition, approximately ten percent of the phylotypes were not characterized previously. Furthermore, phylotypes affiliated with bacterial genera that were not previously found with cultivation-based analyses from the gastric microbiota included e.g. *Caulobacter*, *Actinobacillus*, *Corynebacterium*, *Rothia*, *Gemella*, *Leptotrichia*, *Porphyromonas*, *Capnocytophaga*, *Flexistipes*, *Deinococcus,* and members of the uncultured phylum TM7. *Helicobacter pylori* was the only member of the genus *Helicobacter*, being the most abundant phylotype in *H. pylori* positive subjects[41,44]. The next most abundant genera were *Streptococcus* and *Prevotella*[41,44]. High inter-subject variability of even abundant taxa of the gastric microbiota has been found. Of the 128 phylotypes found in the study of Bik *et al.*[44], 50% were derived from uncultivated bacteria. 67% of these phylotypes have previously been found in the human mouth[22-23]. Moreover, most of the prominent phylotypes (e.g. *Streptococcus, Actinomyces, Prevotella,* and *Gemella*) were also abundant in the throat, suggesting that they may represent swallowed microorganisms from upstream microbiota[41].

1.1.4 Small intestine

The small intestine is the main site of food digestion and nutrient absorption. It is a tubular structure, approximately 3 m long and 2.5–3.5 cm in diameter. It consists of three regions – the duodenum (approximately 25 cm in length), the jejunum (approximately 1.0 m), and the ileum (approximately 2.0 m)[1] (Figure 4). The total absorption area of the small intestine has been calculated to be 200–300 m^2 [46]. However, rapid peristalsis in the small intestine results in a short transit time (3–5 h) for the luminal contents of this region. The pH gradually increases along the small intestine, being 5.7–6.4 in the duodenum, 5.9–6.8 in the jejunum, and 7.3–7.7 in the ileum. In addition, the mucosa itself secretes bicarbonate ions, and thus increases the pH[1].

The duodenum is largely responsible for the enzymatic hydrolysis of food macromolecules. The bulbus duodeni in the beginning of the duodenum analyses the gastric chime and thereafter the rate of emptying of the stomach is regulated via hormonal pathways. Furthermore, bile and pancreatic juice are released in the duodenum. The jejunum is the next part of the small intestine, having a lining specialized in the absorption of carbohydrates and proteins. Most of the absorption takes place in the jejunum.

The ileum, on the other hand, contains abundantly lymphoid tissue. The ileum is specifically responsible for the absorption of vitamin B_{12} and the reabsorption of conjugated bile salts, in addition to absorption of digested products that were not absorbed in the jejunum[46].

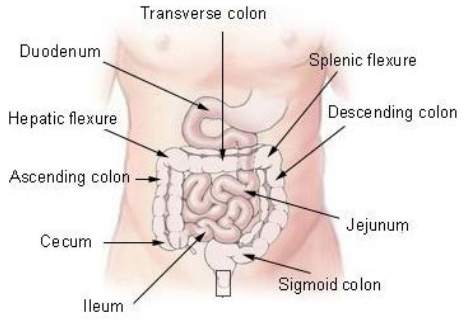

Figure 4. The main anatomical features of the human small intestine and the large intestine (From: SEER Training Modules, Small and large intestine. U.S. National Institutes of Health, National Cancer Institute. May 12th, 2010. (<*http://training.seer.cancer.gov/anatomy/digestive/regions/intestine.html*>)

The number of bacteria residing in the duodenum, jejunum, and ileum is estimated to be 10^4–10^5 cells/g, 10^6–10^7 cells/g, and 10^7–10^8 cells/g, respectively[47]. Factors that limit microbial colonization in the small intestine include flushing action of fluids (drinks, saliva, gastric juices, bile, pancreatic juice, and intestinal fluid), high concentrations of bile salts and proteolytic enzymes (gastric proteases), other antimicrobial defenses such as antimicrobial peptides produced by the intestinal mucosa, and the low pH of the upper region of the small intestine[1]. The common core microbiota detected in ileal effluent has included members of Clostridial cluster I, *Enterococcus* spp., and relatives of the following species: *Oxalobacter formigenes*, *Streptococcus bovis*, *Streptococcus constellatus*, *Streptococcus*

intermedius, *Streptococcus mitis,* and *Veillonella* spp.[47]. The intestinal contents of jejunum and ileum of elderly people have been found to consist of simple, mostly facultatively anaerobic or aerobic microbial communities of streptococci, lactobacilli, members of the Class Gammaproteobacteria, and the *Enterococcus* group. In addition, *Bacteroides* spp. have been found[48]. According to culture-dependent analyses, enterococci, *Escherichia coli*, klebsiella, lactobacilli, staphylococci, and streptococci are present in the jejunum and ileum[49].

1.1.5 Large intestine and feces

The large intestine consists of several regions – cecum, colon (ascending, transverse, descending, and sigmoid), rectum, and anal canal (Figures 1 and 4). The large intestine is approximately 1.5 m long and 6.5 cm in diameter, with a surface area of approximately 1250 cm^2. No digestive enzymes are secreted by the mucosa of the large intestine, and thus further breakdown of dietary constituents is carried out by the resident microbiota[1]. The human colon receives digested food from the ileum. The contents are then mixed and retained for 6–12 h in the cecum and ascending colon. Thereafter, digesta are ejected and pass through the transverse to the descending colon for storage and eventual excretion. Carbohydrates are mainly fermented in the proximal colon, whereas the fermentation of proteins takes place mainly in the distal colon. Transit time of digesta through the colon strongly influences the activities of the gut microbiota. The mean transit time of the orogastrointestinal tract has been reported to be approximately 70 h in UK people consuming a normal daily diet[6,50]. The primary activity of the cecum and colon microbiota is the breakdown of carbohydrates not digested in the ileum to short-chain fatty acids (SCFA), which are then rapidly absorbed. The principal products of carbohydrate fermentation are SCFAs (acetate, propionate, and butyrate), hydrogen and carbon dioxide gases, and bacterial cell mass (biomass)[51]. The amount of energy derived from SCFA accounts for up to 10% of the total energy requirement of humans[52]. From a nutritional point of view, the SCFAs are important since they not only provide the body with energy but are also metabolized in different tissues[51]. The pH of the caecum (~5.7) is lower than that of the ileum because of rapid bacterial fermentation of carbohydrates to short-chain fatty acids (SCFAs). The pH remains similar (5.6–5.7) in the ascending colon and traverse colon, but increases again to 6.6 in the descending and sigmoid colons because of the absorption of SCFAs. In addition, the secretion of bicarbonate by the mucosa increases the pH. The pH in the rectum varies between 6.6 and 6.8[1].

The microbiota in the colon is extremely diverse and on basis of estimations from culture-based and molecular studies more than 1200 prevalent bacterial species altogether reside there. Each individual harbors at least 160 such species[53-54]. However, a large fraction (>80%) of the GI-tract microbiota has not yet been cultured[55], which necessitates the use of molecular techniques in the characterization of GI-tract microbiota. Under normal circumstances, the predominant intestinal microbiota of an adult individual has been reported to be rather stable. However, in studies in which the long-term temporal stability of the predominant microbiota has been assessed from healthy subjects, the number of subjects has been limited[56-59].

Fecal microbiota is dominated by *Eubacterium rectale – Blautia coccoides* group (Erec-group; Clostridial cluster XIV[60]), *Clostridium leptum* group (Clept-group; Clostridial

cluster IV), and Bacteroidetes group[15-16,55, 61- 62], which together account for 60–80% of the fecal bacteria of healthy adults[62-64]. Bacteria belonging to the Erec-group comprise on average 10–45% of the total fecal bacteria as detected with quantitative methods based on hybridization[15-16,62-70], and bacteria belonging to the Clept-group account for 16–27%, thus co-dominating with the members of the Erec-group [62-65,70-72]. Bacteroidetes (*Bacteroides – Prevotella* group) comprise 4–28% of the total fecal bacteria as detected with quantitative hybridization-based methods from healthy adults (probe Bac303)[63-64], whereas the abundance of Bacteroidetes (*Bacteroides – Porphyromonas – Prevotella* group + several Firmicutes) has been found to be ≈ 40% (range 12–64%) using another probe (Bacto 1080)[62,73]. Moreover, the average proportion of *B. fragilis* and *B. vulgatus* species has been found to be 2–9% in healthy adults[63-64,74] and that of *B. fragilis* and *B. distasonis* – using another set of probes – 10–30%[15].

Although found in most humans, *Bifidobacterium* spp. and *Lactobacillus*-group *(*comprising the genera *Lactobacillus, Leuconostoc, Pediococcus,* and *Weissella*) comprise smaller populations among fecal bacteria in adults (bifidobacteria 0.5–6% and lactobacilli <1–3% of the total fecal bacteria)[15-16,62-64,70]. *Atopobium*-group (e.g. genera *Collinsella*, *Eggerthella*, and *Slackia*) also usually comprises 1–5% of the total fecal microbiota. Moreover, there are several bacteria / bacterial groups, which usually comprise less than 1–2% of the total fecal microbiota of healthy adults; these include *Akkermansia muciniphila*, *Clostridium lituseburense* group (Clostridial cluster XI), *Clostridium histolyticum* group (Clostridial clusters I & II), enterobacteria, streptococci, *Veillonella* spp., *Eubacterium cylindroides* group, *Clostridium ramosum* group, sulfate-reducing bacteria, and methanogens[15,62-63,67,75-78]. In addition to bacteria, low levels of viruses, archaea (<1%), and eukaryotes (≤ 2%) are also found in the large intestine[78-80].

1.2 Properties and activities of the human commensal gut microbiota

The human GI-tract, although harboring a vast number of microbes, has only a limited diversity at the phylum level. Microbes from seven bacterial phyla (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, Verromicrobia, and Cyanobacteria-like) and one archael phylum (Euryarchaeota) have been detected in the human intestine[55,61,81-83], whereas microbes from 14 phyla have been found in the oral cavity (Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Cyanobacteria, Euryarchaeota, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, SR1, Synergistetes, Tenericutes, and TM7)[18-19,21]. However, the majority of the GI-tract population are representatives of only two phyla; the Firmicutes and the Bacteroidetes (Figures 5 and 6). Since the current PhD study was mainly focused on fecal samples, oral taxa are only briefly described.

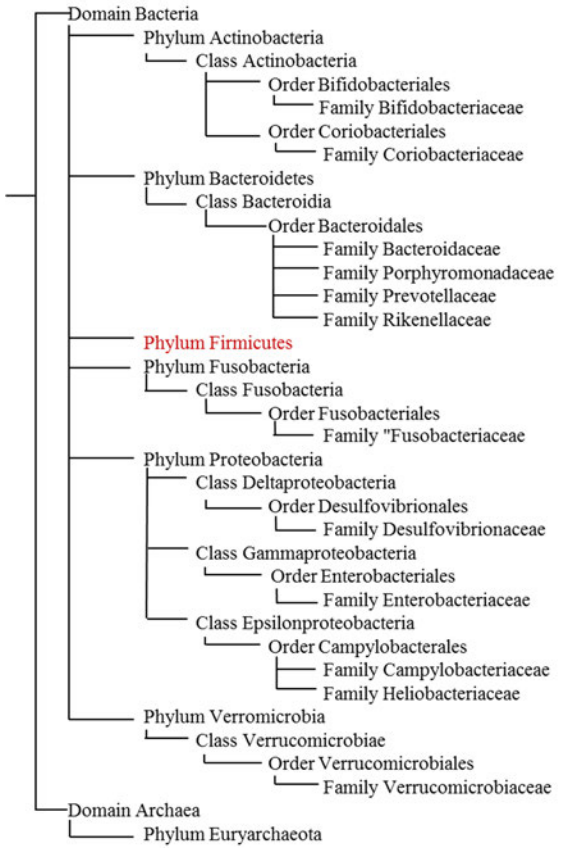

Figure 5. Hierarchy view of the most dominant fecal bacteria. Phylum Firmicutes is depicted in more detail in the following Figure 6.

1.2.1 Phylum Firmicutes

Most of the species within the Phylum Firmicutes are Gram-type positive, although some species stain negative and some genera even possess a true Gram-type negative cell wall containing lipopolysaccharide[84]. The taxonomic and phylogenetic classification of the phylum Firmicutes and especially that of the order Clostridiales are somewhat different. Since the detection of gut microbiota is currently mainly performed with culture-independent methods, and the detection is based on phylogenetic groups, the order Clostridiales is further discussed on the basis of phylogeny (Figure 6).

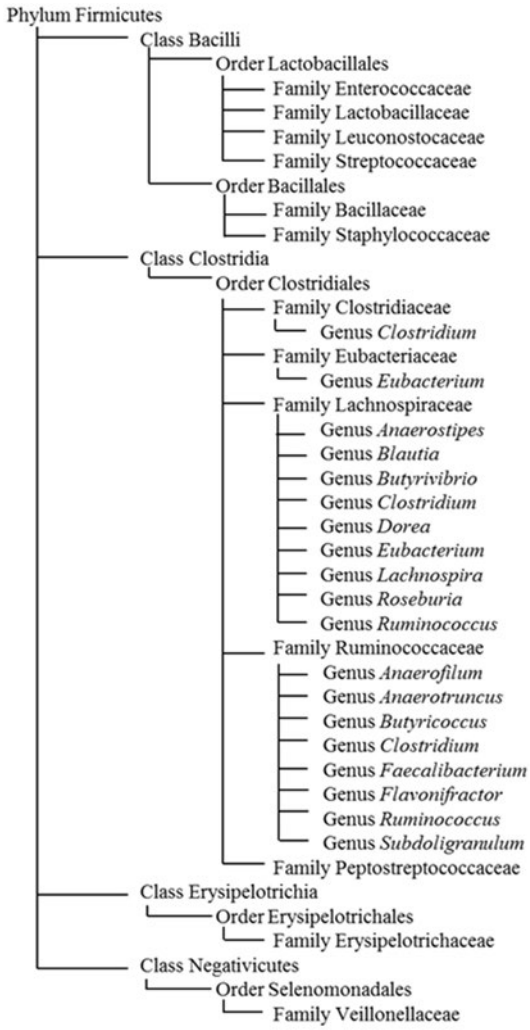

Figure 6. Hierarchy view of the Phylum Firmicutes.

1.2.1.1 Family Clostridiaceae (Clostridial cluster I; "true clostridia")

Clostridiaceae forms the Clostridial cluster I, which is considered to be the "true clostridia" or *Clostridium* sensu stricto. It consists mainly of members of the spore-forming genus *Clostridium*, including the pathogens *Clostridium botulinum*, *Clostridium perfringens*, and *Clostridium tetani*[84-85]. However, members of several other genera, e.g. *Eubacterium* and *Sarcina*, are also phylogenetically placed in Clostridiaceae[86]. The DNA G+C content (mol%) varies between 22 and 53%. The physiology and metabolism of members within Clostridiaceae vary greatly and they are able to utilize many different metabolic pathways (listed below). However, none of the hitherto described *Clostridium* sensu stricto species has been shown to be capable of utilizing O_2 as an electron acceptor for the production of

ATP. Many clostridial species cease growth under oxic conditions, but resume growth once anoxic conditions are reinstated. With regard to carbon, there are chemolithoautotrophic and organoheterotrophic species. The organoheterotrophic species include: 1) saccharolytic species (able to use many or a few monocarbohydrates and one or more polymers such as starch, chitin, xylans, and cellulose); 2) proteolytic and peptidolytic species of amino acid utilizers; 3) lipolytic and purinolytic species; 4) organic acid utilizers; and 5) C-1 compound utilizers. In addition, many of the *Clostridium* sensu stricto species exhibit mixed acid and alcohol fermentations, i.e. they form various concentrations of acetic acid, lactic acid and/or ethanol, propanol and butanol, as well as butyric acid. The amount of butyrate produced is strongly dependent on the growth substrate[84].

1.2.1.2 Family Lachnospiraceae (Clostridial cluster XIV; *Eubacterium rectale – Blautia coccoides* group)

Lachnospiraceae forms the Clostridial cluster XIV, and it includes species of the genera such as *Anaerostipes, Blautia*, *Butyrivibrio, Catonella*, *Clostridium*, *Coprococcus*, *Dorea, Eubacterium*, *Lachnospira*, *Johnsonella*, *Roseburia*, *Ruminococcus*, and *Shuttleworthia*[86]*.* Many members of Lachnospiraceae are polysaccharolytic and produce butyrate. In addition, they are obligately anaerobic. The DNA G+C content (mol%) varies between 29 and 51%.

Anaerostipes spp. produce butyrate, acetate and lactate as major products of glucose metabolism. They are able to produce acids from e.g. fructose, glucose, galactose, fructooligosaccharides, and soluble starch. In addition, they are able to utilize acetate[87]. *Blautia* spp. produce acetate, ethanol, hydrogen, lactate, and succinate as major products of glucose metabolism. They are chemo-organotrophic bacteria, which have a fermentative type of catabolism. Some species use H_2/CO_2 as the major energy source[88]. *Butyrivibrio* spp. are able to ferment a wide range of sugars. In addition, they produce extracellular proteases and both cellular and extracellular esterase activities. Many strains are also pectinolytic and amylolytic. All the characterized *Butyrivibrio fibrisolvens* strains are highly xylanolytic, but only ~10% are capable of growing on cellulose. A few strains are also capable of degrading complex heterocyclic bioflavonoid-type compounds. Due to their wide biochemical diversity, they are presumably involved in the degradation of fibrous plant materials, starches, pectins, and proteins in the caecum and large intestine[89]. *Coprococcus* spp. actively ferment carbohydrates, producing butyric and acetic acids together with formic or propionic and/or lactic acids. Fermentable carbohydrates are either required or are highly stimulatory for growth[90]. The species of the genus *Clostridium* that belong to Lachnospiraceae (e.g. *C. bolteae*, *C. clostridioforme*, *C. hathewayi*, *C. indolis*, *C. jejuense*, *C. nexile*, *C. saccharolyticum*, *C. scindens*, *C. sphenoides*, *C. symbiosum*, and *C. xylanolyticum*) are able to ferment a range of different carbohydrates, producing acetate, lactate, propionate, succinate, formate, butyrate, ethanol, carbon dioxide and/or hydrogen as major products of glucose metabolism. In addition, unlike most of the clostridia *C. symbiosum* is able to ferment glutamate via the α-hydroxyglutarate pathway[91]. *Dorea* spp. produce ethanol, formate, acetate, hydrogen, and carbon dioxide as major products of glucose metabolism; lactate may or may not be formed but butyrate is not produced. *Dorea* spp. are able to utilize a variety of sugars, but e.g. starch is not hydrolyzed[92].

The human-associated species of the genus *Eubacterium* that belong to Lachnospiraceae (*E. cellulosolvens*, *E. eligens*, *E. hallii*, *E. ramulus*, *E. rectale*, and *E. ventriosum*) produce lactate as a major product of glucose metabolism. In addition, some strains produce butyrate, formate, butanol, and/or hydrogen[93]. Furthermore, *E. ramulus* cleaves the ring system of several flavonols and flavones, giving rise to the corresponding hydroxyphenylacetic and hydroxyphenylpropionic acids, respectively, as well as acetate and butyrate. *E. ramulus* is also capable of converting the isoflavonoids genistein and daidzein to the products 2-(4-hydroxyphenyl)-propionic acid and O-desmethylangolensin, respectively[94]. *Lachnospira* spp. are the primary pectin fermenters. In addition to pectin, there is a limited range of carbohydrates that they are able to ferment; sucrose, fructose, and glucose[89]. *Roseburia* spp. are butyrate-producing bacteria that show net acetate utilization during growth on media containing carbohydrates and short-chain fatty acids. Major fermentation products from glucose and acetate include butyrate, lactate, hydrogen and carbon dioxide. However, *Roseburia* spp. are able to ferment only a limited range of sugars[89,95-96]. The species of the genus *Ruminococcus* that belong to Lachnospiraceae (*R. gnavus, R. lactaris, R. obeum, R. torques*) produce lactic, formic, and acetic acids and ethanol, sometimes with trace amounts of succinic and pyruvic acids, as major products of glucose metabolism. However, butyric acid is not formed. In addition, abundant hydrogen is produced. Several strains of *R. gnavus* also form small amounts of ammonia from chopped meat[90,97].

1.2.1.3 Family Ruminococcaceae (Clostridial clusters III and IV)

Ruminococcaceae contains the Clostridial clusters III and IV.

Clostridial cluster IV (*Clostridium leptum* group) includes species of the genera such as *Anaerofilum, Anaerotruncus, Butyricoccus*, *Clostridium, Faecalibacterium, Flavonifractor, Pseudoflavonifractor, Ruminococcus,* and *Subdoligranulum.* Many members of the Clostridial cluster IV are polysaccharolytic and produce butyrate. In addition, they are obligately anaerobic. The DNA G+C content (mol%) varies between 27 and 60%.

Anaerofilum spp. grow chemo-organotrophically on a number of mono- and disaccharides, including glucose and xylose. The principal fermentation products from glucose include lactate, acetate, ethanol, formate, and CO_2, whereas hydrogen is not generated[98]. *Anaerotruncus* spp. produce acetic and butyric acids as end products of glucose metabolism. In addition to glucose, they are able to ferment e.g. fructose, mannose and cellobiose, but there is no growth on e.g. lactose, sorbitol, starch, or xylose. Furthermore, *Anaerotruncus colihominis* is able to utilize e.g. ketobutyric acid, ketovaleric acid, malic acid, and pyruvic acid[99]. Most of the species of the genus *Clostridium* that belong to the Clostridial cluster IV (e.g. *C. leptum*, *C. methylpentosum*, *C. sporosphaeroides*, and *C. viride*) are able to ferment a range of different carbohydrates producing acetate, propionate, butyrate, valerate, n-propanol, carbon dioxide and/or hydrogen as major products of the carbohydrate metabolism. In addition, small amounts of ammonia may occasionally be produced in chopped meat medium. *C. leptum*, however, is not able to ferment glucose or e.g. adonitol, galactose, glycerol, inulin, or sorbose, but ferments maltose. Furthermore, *C. sporosphaeroides* is able to ferment glutamate via the α-hydroxyglutarate pathway and *C. viride* is able to reduce sulfur by a mechanism not linked to energy conservation[91,97, 100-101].

The human-associated species of the genus *Eubacterium* that belong to Ruminococcaceae (*E. desmolans* and *E. siraeum*) produce butyrate, lactate, ethanol, and/or hydrogen as major products of glucose metabolism[93]. In addition, *E. desmolans* is able to degrade steroids[102]. *Faecalibacterium* spp. produce butyrate, formate and d-lactate, but not hydrogen as fermentation products of glucose. They are also able to utilize acetate[103]. *Faecalibacterium prausnitzii* was formerly identified as *Fusobacterium prausnitzii*[104] but was reclassified as *Faecalibacterium prausnitzii* in 2002[103]. The species of the genus *Ruminococcus* that belong to Ruminococcaceae (*R. albus, R. bromii, R. cellulosi, R. callidus, R. flavefaciens*) require a fermentable carbohydrate for good growth and produce acetate, formate, succinate, ethanol, carbon dioxide and/or hydrogen[90,105-106]. *Subdoligranulum variabile* (currently the only species of the genus *Subdoligranulum*) is able to use a variety of different sugars. Esculin is hydrolyzed but starch is not. In addition, cellulose is not degraded. The major products of glucose fermentation are butyric and lactic acids, together with minor amounts of acetic and succinic acids[107]. *Flavonifractor* spp. (*Clostridium orbiscindens* and *Eubacterium plautii* were unified as *Flavonifractor plautii*) are asaccharolytic; glucose, fructose, and ribose are only weakly fermented. Major metabolic end products in trypticase-glucose-yeast extract (TGY) broth are acetate and butyrate. In addition, *Flavonifractor* spp. are able to cleave quercetin and other flavonoids[108]. *Pseudoflavonifractor* spp. (*Bacteroides capillosus* was reclassified as *Pseudoflavonifractor capillosus*) are also asaccharolytic; e.g. glucose, fructose, galactose, and lactose are only weakly fermented. Major metabolic end products in TGY broth are acetate and succinate. Unlike *Flavonifractor* spp. they are unable to cleave quercetin[108].

Clostridial cluster III includes species of the genera such as *Acetivibrio* and *Clostridium.* Many members of the Clostridial cluster III have been isolated from soil or compost environment, and are not usually found in abundance in human samples. Therefore, if Ruminococcaceae is targeted in human samples, mostly members of the Clostridial cluster IV are targeted. Most of the species of the genus *Clostridium* that belong to the Clostridial cluster III (e.g. *C. aldrichii*, *C. cellobioparum*, *C. cellulolyticum*, *C. hungatei*, *C. josui*, *C. stercorarium,* and *C. thermocellum*) are able to ferment a range of different carbohydrates – including cellulose, xylan, and cellobiose – producing acetate, propionate, formate, lactate, butyrate, isovalerate, succinate, ethanol, carbon dioxide, and/or hydrogen as major products of the carbohydrate metabolism[91,109-110].

1.2.1.4 Other relevant Clostridial Families

Family Veillonellaceae. The members of Veillonellaceae are anaerobic bacteria that stain Gram-negative. The most relevant genera of Veillonellaceae with regard to the human oro-gastrointestinal tract are *Anaeroglobus*, *Dialister*, *Megasphaera, Mitsuokella*, *Selenomonas,* and *Veillonella*. *Anaeroglobus* spp. are not able to reduce nitrate or to ferment most of the sugars, although, galactose and mannose are fermented. The metabolic end-products are acetate, propionate, isobutyrate, butyrate, and isovalerate. *Anaeroglobus* spp. mostly reside in the human gastrointestinal tract. *Dialister* spp. are obligately anaerobic or microaerophilic, nonfermentative bacteria that produce acetate, lactate, and propionate as metabolic end products on blood agar. *Dialister* spp. are mainly found in the oral cavity. *Megasphaera eldsdenii*, the only human-associated species of the genus *Megasphaera*, is a chemo-organotrophic bacterium that produces acetate, propionate,

butyrate, iso-butyrate, iso-valerate, 2-methylbutyrate, valerate, carbon dioxide, and/or hydrogen as metabolic end-products of lactate, whereas butyrate, formate, and caproate are produced as the end-products of glucose metabolism. It is not able to reduce nitrate, but produces H_2S. *Mitsuokella* spp. are saccharolytic bacteria that are able to ferment a variety of carbohydrates. The major fermentation end products are acetate, lactate, and succinate. In addition, nitrate is reduced to nitrite. *Mitsuokella* spp. inhabit the human gastrointestinal tract. *Selenomonas* spp. are chemo-organotrophic bacteria that produce acetate, propionate, carbon dioxide and/or lactate as major products of glucose metabolism. In addition, small amounts of hydrogen and succinate may be produced. Furthermore, most of the *Selenomonas* spp. require volatile fatty acids for growth when glucose is used as an energy source. Human *Selenomonas* spp. are mostly associated with the oral cavity. *Veillonella* spp. are generally unable to ferment carbohydrates or amino acids, but reduce nitrate. The major metabolic end-products in TGY-broth are acetate and propionate. In addition, carbon dioxide and hydrogen are produced from lactate. Furthermore, *Veillonella* spp. are able to produce H_2S from e.g. cysteine, thiosulfate, and thioglycolate. *Veillonella* spp. reside mostly in the oral cavity, and in the respiratory and intestinal tracts[111-112].

Family Peptostreptococcaceae (Clostridial cluster XI; *Clostridium lituseburense* group[60, 86]) includes species of the genera such as *Clostridium*, *Eubacterium*, *Filifactor*, and *Peptostreptococcus*. Most of the species of the genus *Clostridium* that belong to Peptostreptococcaceae (e.g. *C. bartlettii*, *C. bifermentans*, *C. difficile*, *C. irregulare*, *C. lituseburense*, and *C. sordellii*) are saccharolytic producing acetate, butyrate, isobutyrate, valerate, and isovalerate as major fermentation products, and formate, propionate, ethanol, propanol, isobutanol, and H_2 as minor products. In addition, *C. bifermentans*, *C. lituseburense*, and *C. sordellii* are also proteolytic. *C. irregulare* is neither saccharolytic nor proteolytic, but gelatin is hydrolyzed. On the other hand, *C. difficile* and *C. sordellii* produce several toxins. *C. difficile* produces toxins A and B; toxin A causes intestinal fluid accumulation and toxin B is primarily cytotoxic. *C. sordellii* also produces two toxins, LT and HT; the major lethal toxin LT is an edema-producing toxin and toxin HT is a hemorrhagic toxin. Moreover, toxin A of *C. difficile* cross-reacts with the HT toxin of *C. sordellii*, whereas toxin B of *C. difficile* cross-reacts with the LT toxin of *C. sordellii* [91,113]. Peptone is the major energy source of *Peptostreptococcus* spp. They do not need carbohydrates for growth, but butyrate and caproate are produced as products of the weak carbohydrate fermentation[106]. Oral saccharolytic *Eubacterium yurii* spp. (produce butyrate, H_2S and indole, in addition to smaller amounts of acetate and propionate) and *Filifactor* spp. (produce acetate, butyrate, isobutyrate, formate, and isovalerate as major fermentation products) have been associated with endodontic infections[60,114].

Family Eubacteriaceae (Clostridial cluster XV; *Eubacterium limosum* group). *Eubacterium limosum*, the type species of genus *Eubacterium*, forms Eubacteriaceae with *Eubacterium aggregans*, *Eubacterium barkeri*, *Eubacterium callenderi*, and *Eubacterium coprostanoligenes* along with species of the genera *Acetobacterium*, *Alkalibacter*, *Anaerofustis*, and *Pseudoramibacter*[60,86]. It has been proposed that the above-mentioned *Eubacterium* species should form the genus *Eubacterium* sensu stricto[115]. The major products of glucose fermentation of *Eubacterium* spp. within Eubacteriaceae are butyrate, caproate, formate, lactate, and/or hydrogen[93]. Moreover, *E. limosum* is nutritionally an extremely versatile species, degrading carbohydrates, amino acids, lactate, and methanol. In addition, it is able to grow autotrophically on H_2 + CO_2 or CO[102], and is able to

produce cobamides and carboxylic acids[93]. Furthermore, *E. coprostanoligenes* is able to reduce cholesterol via the formation of 4-cholesten-3-one, which is then reduced to coprostanol with coprastanone as an intermediate[102].

Family Erysipelotrichaceae contains Clostridial clusters XVI and XVIII[60,86]. **Clostridial cluster XVI** includes species of the genera such as *Clostridium*, *Erysipelothrix*, *Eubacterium*, and *Holdemania*. *Clostridium innocuum* is polysaccharolytic and produces butyrate, lactate, acetate, hydrogen, and carbon dioxide as end products of carbohydrate fermentation[91]. *Erysipelothrix* spp. are chemo-organotrophic; metabolism is respiratory and weakly fermentative. Acid, but no gas, is produced from glucose and other carbohydrates[116]. One of the major clusters of *Eubacterium* spp. includes species within Erysipelotrichaceae (*E. biforme*, *E. cylindroides*, *E. dolichum*, and *E. tortuosum*). The main products of glucose fermentation of *Eubacterium* spp. within Erysipelotrichaceae are butyrate, caproate, formate, lactate, and/or hydrogen[91,102]. *Holdemania* spp. produce acetic and lactic acids as main fermentation end products from glucose, whereas succinic acid is produced only in smaller amounts[117]. **Clostridial cluster XVIII** (*Clostridium ramosum* group[60]) consists of helically coiled *Clostridium cocleatum*, *Clostridium ramosum*, *Clostridium saccharogumia,* and *Clostridium spiroforme* species that are isolated from the gastrointestinal tract. They are saccharolytic and produce acetate, formate, lactate, and succinate but not butyrate as major fermentation products. In addition, *C. saccharogumia* has been shown to be involved in the conversion of the plant lignan secoisolariciresinol diglucoside. Furthermore, *C. ramosum* has been reported to produce β-lactamase and *C. spiroforme* to produce a toxin similar to the *C. perfringens* type E iota toxin[91,118-119].

1.2.1.5 Class Bacilli

Order Lactobacillales includes Families such as Enterococcaceae, Lactobacillaceae, Leuconostocaceae, and Streptococcaceae and are commonly referred to as lactic acid bacteria (LAB). They are a diverse group of Gram-positive, non-spore forming, facultatively anaerobic bacteria that have a G+C content of less than 50 mol-% and produce lactic acid by fermentation. Their nutritional requirements are complex and variable [120]. ***Enterococcus*** spp. of Enterococcaceae are chemo-organotrophic bacteria that are able to ferment a wide variety of substrates and have a homofermentative lactic acid fermentation. The predominant product of glucose fermentation is L(+)-lactic acid. However, under aerobic conditions, *Enterococcus faecalis* ferments glucose to acetic acid, acetoin, and carbon dioxide. Furthermore, if the fermentation takes place under neutral to slightly alkaline conditions glucose is fermented to formate, ethanol, and acetate. *Enterococcus* spp. harbor a wide variety of plasmids and transposons that are involved e.g. in the transfer of antibiotic resistance and virulence factor determinants. In addition, the *Enterococcus* spp. are resistant to 40% (v/v) bile. Although enterococci are mostly commensal human bacteria, they have been increasingly isolated from a variety of nosocomial and other infections[120-121]. Lactobacillaceae includes genera such as *Lactobacillus* and *Pediococcus*. ***Lactobacillus*** spp. are aciduric or acidophilic bacteria that are obligately saccharoclastic. With glucose as a carbon source, *Lactobacillus* spp. may be either homofermentative (>85% of the end product is lactic acid) or heterofermentative (production of lactic acid, CO_2, ethanol and/or acetic acid). In addition, under aerobic conditions increased amounts of acetic acid may be produced at the expense of lactic acid or ethanol.

Due to the heterogeneous fermentation behavior, *Lactobacillus* spp. may be divided into three subgroups: A) Obligately homofermentative lactobacilli, in which hexoses are almost exclusively (>85%) fermented to lactate (e.g. *L. acidophilus*, *L. crispatus*, *L. delbrueckii*, *L. gasseri*, *L. johnsonii*, and *L. salivarius*); B) Facultatively heterofermentative lactobacilli, in which hexoses are almost exclusively fermented to lactate, but under glucose limitation lactic acid, acetic acid, ethanol, and formic acid are produced. Moreover, availability of the nutrients, pH and redox potential all have an effect on which end products will be formed (e.g. *L. casei*, *L. paracasei*, *L. rhamnosus*, and *L. plantarum*); C) Obligately heterofermentative lactobacilli, in which hexoses are fermented to lactic acid, ethanol, acetic acid and CO_2 (e.g. *L. brevis*, *L. buchneri*, *L. fermentum*, *L. oris*, and *L. reuteri*)[120,122]. ***Pediococcus*** spp. are able to ferment a wide variety of substrates. Under anaerobic conditions, *Pediococcus* spp. ferment glucose to lactic acid. Pediococci are the only lactic acid bacteria that divide alternately in two perpendicular directions to form tetrads. They are mostly found in various beverages, and unlike enterococci and lactobacilli only rarely in the GIT[120,122]. Leuconostocaceae includes genera such as *Leuconostoc* and *Weissella*. ***Leuconostoc*** spp. are nonacidophilic, chemo-organotrophic, heterofermentative bacteria that are mostly found in food and drink products[120,123]. ***Weissella*** spp. are chemo-organotrophic, heterofermentative bacteria that are able to ferment a wide variety of substrates. The end products of glucose metabolism are DL- or D-lactate, ethanol, CO_2 and/or acetate. *Weissella confusa* forms part of the commensal human microbiota, whereas other *Weissella* spp. are mostly associated with various food products[120, 123]. Streptococcaceae includes genera such as *Lactococcus* and *Streptococcus*. ***Lactococcus*** spp. are chemo-organotrophic, homofermentative bacteria that are able to ferment a variety of substrates. They are commonly found in plant material and from dairy products, but only transiently in the GIT[120-121]. ***Streptococcus*** spp. are chemo-organotrophic, homofermentative bacteria that are able to ferment a wide range of substrates. However, under glucose-limited conditions, other end products such as formate, acetate, and ethanol, may also be detected. Many of the known species are commensals in humans, although some are important pathogens (e.g. *S. pyogenes* and *S. pneumoniae*), and some are used for production of dairy products (*S. thermophilus* for production of yoghurt)[120-121].

Order Bacillales consists of e.g. Families Bacillaceae and Staphylococcaceae. ***Bacillus*** spp. of Bacillaceae are mostly aerobic or facultatively anaerobic bacteria, although a few species have been described to be strictly anaerobic. They are mostly chemo-organotrophic – two species are facultative chemolithotrophs – spore-forming bacteria. *Bacillus* spp. have a wide range of physiological abilities ranging from psychrophilic to thermophilic, and acidophilic to alkaliphilic. Furthermore, some strains are salt tolerant and some are halophilic. Most species are able to use glucose and/or other fermentable carbohydrates, but some species are not able to utilize carbohydrates at all. In addition, some species are able to decompose pectin and polysaccharides. *Bacillus* spp. are also able to use both inorganic and organic sources of nitrogen, and able to reduce nitrate to nitrite. Moreover, *Bacillus cereus* is an important foodborne pathogen, which produces enterotoxins and some strains produce an emetic toxin (cereulide)[124-125]. ***Staphylococcus*** spp. of Staphylococcaceae are facultatively anaerobic, chemo-organotrophic bacteria. The main fermentation product of most of the species is lactic acid. However, under aerobic conditions acetic acid and carbon dioxide are the main end-products. Nutritional requirements of *Staphylococcus* spp. are variable; however, most of the species require

an organic source of nitrogen. *Staphylococcus* spp. are mainly associated with skin, skin glands, and mucus membranes. Some species are opportunistic pathogens, most notably *S. aureus*, which produces enterotoxins[125-126].

1.2.2 Phylum Bacteroidetes

The members of the Phylum Bacteroidetes are Gram-negative bacteria, having a DNA G+C content (mol%) of 28–61%. Most members of the Phylum Bacteroidetes were originally classified as part of the genus *Bacteroides*. Within the past few decades, the taxonomy of the genus *Bacteroides* has undergone major revisions and many new genera have been identified.

1.2.2.1 Family Bacteroidaceae

Bacteroides spp. are saccharolytic bacteria that contain a wealth of polysaccharide-degrading enzymes. They have an excellent ability to ferment simple and complex sugars and polysaccharides, such as xylans, noncellulosic glucans, pectins, galactomannans, arabinogalactans, mucopolysaccharides, and mucin, producing acetate and succinate as major metabolic end products. In addition, most species are weakly proteolytic. Peptidases produced by *Bacteroides* spp. facilitate the degradation of proteins to yield a variety of free amino acids and peptides. Furthermore, *Bacteroides* spp. have an enriched set of glycan, vitamin, and cofactor enzymes important for food digestion. Moreover, *Bacteroides fragilis* plays a key role in the enterohepatic circulation of bile acids by helping the process of biotransformation between conjugated and deconjugated bile salts. The DNA G+C content (mol%) of *Bacteroides* spp. is 40–48%[127-132]. *Bacteroides* spp. differ significantly from other Bacteroidaceae species in possessing enzymes of the hexose monophosphate shunt-pentose phosphate pathway**.** *B. fragilis* and related species also possess sphingolipids, predominantly methyl-branched long-chain fatty acids, and menaquinones as the sole respiratory quinones[131]. The completion of whole genome sequencing projects has shed more light onto the ecophysiology of *Bacteroides* spp.; they have been shown to possess 1) complex systems to sense the nutrient availability and tailor nutrient-metabolizing systems accordingly; 2) multiple pump systems to rid the bacteria of toxic substances; and 3) the ability to control the environment by interacting with the host immune system so that it controls other, possibly competing, pathogenic bacteria[127]. Furthermore, it has been shown in mice studies that *Bacteroides thetaiotaomicron* is able to redirect its carbohydrate-utilizing capability from dietary to host polysaccharides according to nutrient availability[133].

According to the all-species living tree, the genus *Parabacteroides* belongs to Bacteroidaceae[86], whereas according to the NCBI taxonomy *Parabacteroides* is placed within Porphyromonadaceae[134]. *Parabacteroides* spp. were moved away from the genus *Bacteroides* because of clear differences in the 16S rRNA-based phylogeny (e.g. *Bacteroides merdae* → *Parabacteroides merdae* and *Bacteroides distasonis* → *Parabacteroides distasonis*). However, at the whole genome level, the *Parabacteroides* spp. are a part of the *Bacteroides* genus[130]. The physiological properties of the genus *Parabacteroides* resemble those of the genus *Bacteroides*. *Parabacteroides* spp. are saccharolytic bacteria that produce acetate and succinate as major metabolic end products. The G+C content is 43–46 mol%[135].

1.2.2.2 Families Prevotellaceae, Porphyromonadaceae and Rikenellaceae

Most of the bacteria within Prevotellaceae and Porphyromonadaceae have been isolated from the oral cavity and are commonly found in oral sites in culture-based studies, whereas with DNA-based molecular studies members of Prevotellaceae and Porphyromonadaceae have also been found in feces. **Family Prevotellaceae** includes genera such as *Prevotella* and *Paraprevotella*[86,128]. *Prevotella* spp., which are mainly associated with the oral cavity, are pigmented, moderately saccharolytic bacteria. They mainly produce acetic and succinic acids as major products of glucose fermentation. The G+C content is 40–60 mol%[128-129]. *Paraprevotella* spp., which have been isolated from feces, are saccharolytic and able to utilize various sugars, producing succinic and acetic acids as end products of glucose metabolism. The DNA G+C content is 48–49 mol%[136]. **Family Porphyromonadaceae** includes oral genera such as *Porphyromonas* and *Tannerella*, which are asaccharolytic, pigmented, and proteolytic bacteria. The DNA G+C content ranges between 46 and 54 mol%[86,137-138].

Family Rikenellaceae consists of the gastrointestinal genera *Alistipes* and *Rikenella* [86] that have high mol% G+C of DNA (55–61%). Most *Alistipes* spp. are saccharolytic bacteria. The major glucose fermentation product is succinic acid, together with minor amounts of acetic, isovaleric and propionic acids; lactic acid, isobutyric and butyric acids are sometimes produced in very small amounts[139-140]. *Rikenella* spp. are weakly saccharolytic bacteria that produce acetate and succinate as the end products of carbohydrate fermentation[141].

1.2.3 Phylum Actinobacteria

Phylum Actinobacteria consists of Gram-positive bacterial genera that mostly have DNA of high guanine + cytosine (G + C) content (60–64%), with the exception of the genus *Atopobium* with a G + C range of approx. 36–46 mol%.

1.2.3.1 Family Bifidobacteriaceae

Bifidobacterium spp. are proteolytic, acid-tolerant (but not acidophilic) bacteria that produce acetate and lactate from sugars, and are able to hydrolyze a wide range of polysaccharides. Bifidobacteria are common inhabitants in the human intestine. The most commonly found gastrointestinal bifidobacteria include; *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. longum*, and *B. pseudocatenulatum*[142-145]. *B. dentium* is the only *Bifidobacterium* that has been isolated from the human oral cavity[146]. The other oral genera within Bifidobacteriaceae include *Scardovia* and *Parascardovia*. *Scardovia inopinata* (formerly *B. inopinatum*) and *Parascardovia denticolens* (formerly *B. denticolens*) have been transferred from the genus *Bifidobacterium* since they have distinct phenotypic characteristics and low G+C contents as compared to the bifidobacteria[29]. *Scardovia* spp. and *Parascardovia* spp. are non-acid-fast and saccharoclastic bacteria. The G-C content of the *Scardovia* spp. is ~45 mol% and that of *Parascardovia* spp. ~55 mol%[29].

1.2.3.2 Family Coriobacteriaceae

Coriobacteriaceae was formed on the basis of reclassification of former members of the genus *Eubacterium*. It consists of members of the genera such as *Atopobium, Collinsella, Coriobacterium*, *Eggerthella, Olsenella,* and *Slackia*[86]. Genera *Collinsella, Coriobacterium*, and *Eggerthella* are associated with the gastrointestinal tract, whereas *Atopobium, Olsenella,* and *Slackia* are mostly found in the oral cavity[147-149]. *Atopobium* spp. produce major amounts of lactic acid as fermentation products[148,150]. *Coriobacterium* spp. produce acetate, lactate, ethanol, CO_2 and/or H_2 as major products of glucose fermentation[151]. *Collinsella* spp. produce lactate, formate, ethanol, and H_2 as principal fermentation products from glucose. In addition, the cell wall contains a unique A4β-type peptidoglycan[93, 152]. *Eggerthella* spp. produce acetate, lactate, and succinate as principal fermentation products from glucose. *Eggerthella lenta* is capable of inactivating digoxin, a widely used cardiac glycoside. In addition, *E. lenta*-like organisms have been found to be able to deconjugate both glycine and taurine conjugates of cholic aid and chenodeoxycholic acid and they are found to be quite active in transforming steroid compounds[93,147,149]. The type species of the genus *Slackia*, *Slackia exigua*, does not ferment sugars. However, *Slackia heliotrinireducens* produces acetate as a fermentation product from glucose[147].

1.2.4 Some other predominant human bacterial Phyla

The most important members of **Class Delta-Proteobacteria** of **Phylum Proteobacteria** with regard to the lower human gastro-intestinal tract are the H_2S producing bacteria of Family Desulfovibrionaceae (e.g. *Desulfovibrio* spp. and *Bilophila* spp.). Sulfate-reducing bacteria (SRB) are a morphologically and physiologically diverse group of strictly anaerobic organisms, which share the ability to dissimilate sulfate to sulfide while oxidizing various growth substrates. *Desulfovibrio* spp. (namely *D. desulfuricans* subsp. *desulfuricans*, *D. fairfieldensis, D. piger,* and *D. vulgaris*) are non-saccharolytic, non-proteolytic bacteria that contain desulfoviridin. *Desulfovibrio* spp. commonly utilize lactate, ethanol, and hydrogen as electron donors for sulfate reduction. However, a wide range of different compounds such as short-chain fatty acids, other carboxylic acids, alcohols, sugars, amino acids, and aromatic compounds, may also act as electron donors for dissimilatory sulfate reduction. They are not able to grow with acetate as an electron donor, because of incomplete oxidization of organic compounds to acetate. In addition, they use sulfate, sulfite, and thiosulfate and do not use nitrite as electron acceptors. The activities of SRB are controlled largely by the availability of oxidized sulfur compounds, especially SO_4^{2-} and SO_3^{2-}, which may be present in drinking water or/and foods as preservatives. Intestinal sulfate can also be derived from endogenous sources such as sulfated mucins (sulfomucins), sulfate-conjugated bile, and chondroitin sulfate. Furthermore, the use of chemically bound, endogenous sulfate by SRB is facilitated through interactions with sulfatase-harboring bacteria (e.g. *Bacteroides* spp.)[51,78,153-155]. *Bilophila* spp. are non-saccharolytic bacteria, which produce acetic acid as major and succinic and lactic acids as minor fermentation products from peptone-yeast broth. In addition, ammonia is produced from urea and nitrate is reduced to nitrite and occasionally to N_2. Moreover, desulfoviridin is weakly present in some strains, but sulfate is not reduced. Besides not being able to reduce sulfate, *Bilophila* spp. also differs from the other human-associated members of

Desulfovibrionaceae in other ways; the G+C-content of *Bilophila* spp. is 40 mol%[156], whereas that of human *Desulfovibrio* spp. ranges between 59 and 64 mol%[154].

Class Gammaproteobacteria of **Phylum Proteobacteria** contains e.g. Family Enterobacteriaceae, which includes several enterobacterial genera (e.g. *Cronobacter, Enterobacter*, *Escherichia*, *Klebsiella*, *Salmonella*, *Shigella*, and *Yersinia*). *Salmonella* spp., *Shigella* spp., *Yersinia* spp., and many *Escherichia coli* types (EHEC, ETEC etc.) are widely recognized enteric pathogens, whereas other *Escherichia* spp., *Enterobacter* spp. and *Klebsiella* spp. are opportunistic human pathogens. *Enterobacter* spp. are facultatively anaerobic polysaccharolytic bacteria that produce CO_2 and H_2 as major fermentation products of glucose. In addition, some *Enterobacter* spp. are able to fix nitrogen. Most *Cronobacter* and *Enterobacter* bacteremias are acquired in hospitals. The majority of the infections are caused by *E. cloacae*. *E. agglomerans* and *Cronobacter sakazakii* (former *Enterobacter sakazakii*) are the next most prevalent causative agents. Moreover, *C. sakazakii* has been found on multiple occasions in infant milk powder, where it has caused meningitis and generalized sepsis. The increased prevalence of nosocomial infections may be due to increased resistance to antimicrobial agents and disinfectants[157]. *Escherichia* spp. are aerobic and facultatively anaerobic chemo-organotrophic, polysaccharolytic bacteria that produce lactic, acetic, and formic acids and usually CO_2 and H_2 (1:1) as fermentation products of glucose. In addition to fermentative metabolism, *Escherichia* spp. also have respiratory metabolism. *E. coli* is a normal part of the human gut microbiota. Most *E. coli* strains are non-pathogenic, but certain serotypes play an important role in intestinal and other diseases, e.g.: 1) Enteropathogenic *E. coli* (EPEC) are diarrheagenic *E. coli,* the pathogenic mechanisms of which have not yet been demonstrated to be related to heat-labile enterotoxins or heat-stable enterotoxins or *Shigella*-like invasiveness. In addition, EPEC are not invasive in the same way as *Salmonella*, *Shigella*, and *Yersinia*; 2) Enterotoxigenic *E. coli* (ETEC) do not invade epithelial cells, but produce one or more enterotoxins that are either heat-labile or heat-stable; 3) Enteroinvasive *E. coli* (EIEC) are, like Shigella, able to invade and multiply in the intestinal epithelial cells in the colon; 4) Shiga toxin producing (STEC) or Vero cytotoxin-producing (VTEC) *E. coli* have the ability to produce either one or both of at least two antigenically distinct cytotoxins. Enterohemorrhagic *E. coli* (EHEC) is used to describe a subgroup O157:H7 of STEC/VTEC that causes hemorrhagic colitis[158]. *Shigella* spp. are facultatively anaerobic, chemo-organotrophic bacteria that have both a respiratory and a fermentative type of metabolism and are able to reduce nitrates to nitrites. They produce pyruvate, which is further converted into formic acid, acetic acid, and ethanol, as a fermentation product from glucose. However, gas is not produced as a fermentation product. *Shigella* spp. are human intestinal pathogens that cause shigellosis (bacillary dysentery). Common clinical signs are fever, severe abdominal pain, and cramping. In addition, strains producing shiga-toxin may cause hemolytic uremic syndrome. *Shigella* spp. are acid-resistant. Moreover, stationary phase cells of *S. flexneri* are able to survive for several hours at pH 2.5, which is thought to contribute to the low infective dose of *Shigella* spp. (as low as 10–100 organisms). *Shigella* spp. penetrate into the colon epithelial cells, multiply in them and thereafter spread from cell to cell through the mucosa[159]. *Yersinia* spp. are facultatively anaerobic bacteria that have both a respiratory and a fermentative type of metabolism and are mostly able to reduce nitrate to nitrite. Little or no gas is produced as a fermentation product. *Y. pestis* is the causative agent of plague, which is primarily a disease of wild

rodents. Flea bites and ingestion of infected animal tissue transmit *Y. pestis* among wild rodents. If no other hosts are available, fleas may transmit *Y. pestis* to humans. Untreated plague evolves in 5–10 days to profound septicemia. *Y. enterocolitica* is another human pathogen, which mostly causes acute enteritis in children. In adults, the secondary clinical consequences also include arthritis and erythema nodosum[160]. *Klebsiella* spp. are facultatively anaerobic polysaccharolytic bacteria that produce 2,3-butanediol, CO_2, and H_2 as major glucose fermentation products. In addition, lactic, acetic, and formic acids are formed in smaller amounts. As well as fermentative metabolism, *Klebsiella* spp. also exhibit respiratory metabolism. Furthermore, most strains hydrolyze urea and β-galactosides, and some strains are able to fix nitrogen. Human *Klebsiella* infections are mainly caused by *K. pneumoniae* and *K. oxytoca*. Four components of *Klebsiella* have long been implicated in the pathogenesis: adhesins, capsular polysaccharides, lipopolysaccharide (LPS), and iron-scavenging systems (siderophores)[161]. *Salmonella* spp. are chemo-organotrophic bacteria having both fermentative and oxidative metabolism. They ferment glucose to formate, ethanol, acetate, or lactate. The electron transport chain is cytochrome-based with oxygen, nitrate, fumarate, dimethyl sulfoxide, and trimethylamine oxide as terminal electron acceptors. *Salmonella* spp. usually cannot metabolize lactose or sucrose, but are able to hydrolyze 4-methylumbelliferyl caprylate, and produce H_2S from sulfur-containing amino acids. Moreover, the genes for production of hydrogen sulfide can be used as *Salmonella*-specific molecular markers. In addition, most serotypes of *Salmonella* are prototrophs[162].

Class Epsilonproteobacteria of **Phylum Proteobacteria** contains e.g. Families Campylobacteriaceae (e.g. *Campylobacter* spp.) and Helicobacteriaceae (e.g. *Helicobacter* spp.). *Campylobacter* spp. are chemo-organotrophic bacteria, with a respiratory type of metabolism. Energy is obtained from amino acids or tricarboxylic acid cycle intermediates, whereas carbohydrates are neither fermented nor oxidized. Most species are also able to reduce nitrate. Most of the *Campylobacter* spp. are human pathogens. *Campylobacter jejuni* subsp. *jejuni* is regarded as the most common bacterial cause of gastroenteritis in humans worldwide. It is more common in children than in adults, and more prevalent in summer and fall than in winter and spring[163]. *Helicobacter* spp. are chemo-organotrophic bacteria, with a respiratory type of metabolism. Energy is obtained from amino acids, whereas carbohydrates are neither fermented nor oxidized when standard methods are used for testing of carbohydrate metabolism. However, genome analysis of *Helicobacter pylori* has revealed that *H. pylori* possesses catabolic pathways for glucose, and may therefore in theory be able to utilize glucose. Many *Helicobacter* spp. are human or animal pathogens. *H. pylori* is the leading microbiological cause of human gastric and duodenal ulcers. In addition, *H. pylori* infections have been associated with the development of atrophic gastritis and subsequent progression to gastric cancer, mucosa-associated lymphoid tissue lymphoma, adenocarcinoma of the distal stomach, and Ménétrièr's disease (a disorder in which the gastric folds become hypertrophic). Moreover, *H. pylori* has been classified as a class 1 carcinogen by the International Agency for Research in Cancer of the World Health Organization[164].

Most of the members of the **Phylum Verrumicrobia** are marine bacteria. However, *Akkermansia muciniphila* has been found in the human intestine. *A. muciniphila* is a chemo-organotrophic bacterium, which is able to use mucin as a carbon, energy, and nitrogen source. It can grow on a limited number of carbohydrates, including glucose, but

only when a protein source is present. Moreover, it is also able to release sulfate in a free form from mucin fermentation[165].

Phylum Fusobacteria is formed of the Families Fusobacteriaceae and Leptotrichiaceae. Fusobacteriaceae are found from both the oral cavity and gastrointestinal tract, whereas Leptotrichiaceae are mostly associated with the oral cavity. *Fusobacterium* spp. form butyric acid as a major end product from peptone or glucose (without isobutyric or isovaleric acids). Peptides are the most important energy source. *Fusobacterium* spp. are either non-fermentative or only weakly fermentative. The utilization of glucose is dependent on energy supplied by fermentation of amino acids. *F. necrophorum* is also able to deconjugate bile salts. Moreover, *F. nucleatum* and *F. necrophorum*, as well as other *Fusobacterium* species, possess a cell wall lipopolysaccharide with the characteristics of an endotoxin[166]. *Leptotrichia* spp. are saccharolytic bacteria, which produce lactic acid as the major fermentation product. In addition, trace amounts of hydrogen sulfide are also produced[167].

1.2.5 Domain Archaea, phylum Euryarchaeota

Methanogenic Archaea (MA) are a phylogenetically diverse group of strictly anaerobic Euryarchaeota with an energy metabolism that is restricted to the formation of methane from various components[168]. In the human GI-tract MA metabolize major fermentation products, such as alcohols, short chain organic acids, CO_2, and H_2[52,169]. The diversity of MA in the human gut is scarce. The most abundant MA, *Methanobrevibacter smithii*, converts H_2, CO_2, and formate into CH_4, using carbon as the terminal electron acceptor[170-171]. *M. smithii* can remove fermentation end products, such as methanol and ethanol produced by other bacteria. The energy metabolism of the other human MA, *Methanosphaera stadtmanae,* is limited to using H_2 to reduce methanol to methane. In addition, it is dependent on acetate as a carbon source[172]. SRB and methanogens are largely mutually exclusive in the colon (UK; 70% SRB, 30% MA)[78]. However, under conditions of sulfate availability SRB are able to outcompete methanogenic bacteria for H_2[173].

1.2.6 The importance of the metabolic activities of the gut microbiota from the host's perspective

From the host's perspective, there are numerous activities of the commensal gut microbiota that are of great importance to health. Carbohydrates are mainly fermented in the proximal colon, whereas the fermentation of proteins takes place mainly in the distal colon. However, the metabolic output of the microbial community depends not only on available substrates, but also on the gut environment, with the pH playing major role. For example at pH 6.7 *Bacteroides* spp. predominate, whereas at pH 5.5 bacteria related to *E. rectale* predominate[112,174-175]. The main saccharolytic genera in the human gastrointestinal tract are *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Lactobacillus*, and *Ruminococcus*. They are able to produce SCFA, which have both local and systematic beneficial biological effects (Chapter 1.4.4.1 Dietary fiber). A wide range of bacteria have proteolytic activities, such as clostridia, *Propionibacterium* spp., *Prevotella* spp., *Bifidobacterium* spp., and *Bacteroides* spp. The protein metabolism, however, is not as favorable to the host as the carbohydrate metabolism; some of the end-products of amino

acid metabolism may be deleterious to the host, e.g. ammonia, amines, and phenol compounds. Some species of the genera *Bacteroides*, ruminococci, and *Akkermansia* are able to break down mucin. Moreover, several *Eubacterium* spp. and *Clostridium* spp. are able to dehydroxylate bile acids, some *Clostridium* spp. transform conjugated bilirubin, *Eubacterium coprostanoligenes* is able to convert cholesterol to coprostanol and *Bacteroides* spp. inactivate tryptic activity, have dipeptidase activity, and play a key role in the enterohepatic circulation of bile acids[6,102,127-130,165].

In addition to the individual activities, cross-feeding between the gut microbes and the metabolic networks thus created are also of great importance. For example, it has been shown *in vitro* that lactate produced by *Bifidobacterium adolescentis* as a fermentation product from fructo-oligosaccharide (FOS) and starch was further utilized by butyrate producers, which were not able to grow solely on FOS and starch[176]. In addition, *Roseburia intestinalis* and *Anaerostipes caccae* have been shown to be able to grow with *Bifidobacterium longum* using FOS; *R. intestinalis* was able to grow on FOS-supplemented medium when acetate, a major fermentation product of *B. longum*, was added to the medium, whereas *A. caccae* was able to utilize fructose which was released during the bifidobacterial fermentation of FOS[177]. Besides other survival mechanisms, gene transfer within and from outside the gut microbiota has also been shown to occur. In Japan, where consumption of marine algae is high, a Japanese gut bacterium (*Bacteroides plebeius*) has been shown to acquire genes coding for porphyromonases, agarases, and associated proteins and thus the ability to utilize marine algae. These algae are not readily fermentable by Western gut microbiota[178].

1.3 Methods used for studying the gastrointestinal microbiota

The application of molecular techniques for the identification and/or enumeration of different microbial groups has increased enormously during the past decade. Especially the 16S ribosomal RNA (rRNA)-based detection and enumeration techniques have advanced greatly. Molecular techniques enable faster detection and enumeration of specific bacteria than conventional culture-base methods. However, culture-based methods are still the only validated methods able to quantitate viable bacteria. When molecular techniques are applied to study microbial communities of the GI-tract, it should be kept in mind that the human oro-GI-tract is an open system – i.e. oral bacteria may be detected with molecular techniques from feces as well. Therefore, it is of the utmost importance to understand the limitations – as well as the advantages – related to the techniques applied (Figure 7).

In addition to the analysis techniques applied, sampling and transportation may also affect the quality of the samples. Conditions during sample transportation have a major impact on sample quality and therefore the time between sampling and further processing and storing should be limited to the minimum. It should be kept in mind that regardless of how sophisticated the applied method for characterization of the microbial community is, the result can be only as good as the sample which arrives in the laboratory. Furthermore, the sample processing method must be chosen taking into account both the properties of the target microbial groups and the method used in the subsequent detection step.

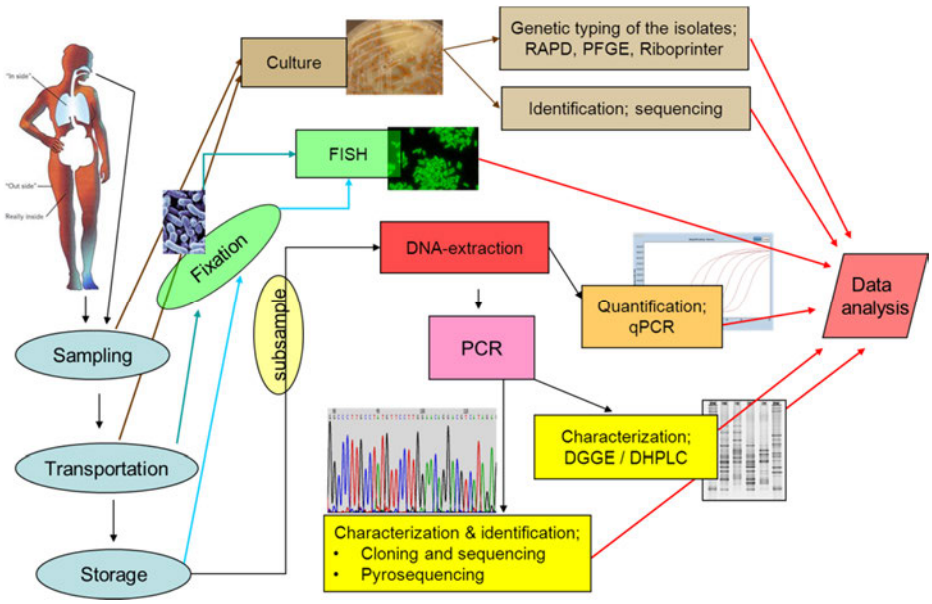

Figure 7. Microbiological analysis of human samples.

1.3.1 Microscopy

There are several types of microscopy that may be used for the study of microbial communities. Light microscopy is nowadays used mainly for verification of the purity of bacterial isolates or for studying the effects of gram- or spore-staining, whereas fluorescence microscopy has widely been used in microbial ecology. Epifluorescence microscopy (two-dimensional) and confocal scanning laser microscopy (CSLM; three-dimensional) are used for studying specimens that fluoresce. Furthermore, CSLM allows a detailed, non-destructive examination of thick microbial samples. The advantages of fluorescence microscopy are rapidity, easy usability, and visualization of spatial distribution of cells. With a suitable combination of fluorescent stains, the differentiation between viable and dead cells is possible[179-180]. Transmission electron microscopy (TEM), scanning electron microscopy (SEM), and atomic force microscopy (AFM) allow higher resolution of the cell structure than the light-based microscopies. In TEM and SEM, electrons are used for imaging instead of light. The resolution of TEM enables the imaging of molecules (proteins, nucleic acids etc.). However, since electrons are only able to penetrate extra thin layers, ultra-thin specimens are needed; e.g. several thin layers are needed for a single bacterium[181]. In SEM, information about the sample's external morphology, chemical composition, crystalline structure, and orientation of materials making up the sample may be obtained. In addition, layering is not needed. The sample is coated with a thin layer of conducting material, commonly carbon, gold, or some other metal or alloy, and thereafter analyzed from above[182]. AFM allows the investigation of surfaces on an atomic scale. Images are obtained by measurement of the force on a sharp tip created by the proximity to the surface of the sample. The force is kept small and at a constant level with a feedback mechanism[183].

1.3.2 Culturing

An ideal method for studying microbial communities would detect and enumerate all microbial species present in the samples with equal efficiency. It has been speculated that many microbial communities are too diverse to be counted exhaustively, which has led to the application of statistical approaches for estimation of the diversity[184]. Microbial community analysis by cultivation is extremely laborious, especially when complex samples with high diversity are studied. If cultivation is used for microbial community analysis, several non-selective and selective culture media and different growth conditions including different temperatures and atmospheres, followed by accurate identification of a large number of isolates from each medium, should be included in order to obtain the best possible overview of the diversity of the microbial population. The dominant cultivable population is recovered from non-selective media, whereas selective media allow detection of groups or species that are present in lower numbers. Identification of isolates with traditional methods is based on the assessment of several phenotypic features, which is often inaccurate, and may lead to underestimation of the species diversity. Currently, this is combined with molecular identification techniques, e.g. sequencing.

A large fraction (>80%) of the GI-tract microbiota has not yet been cultured[55], partly due to the poor ability of the routinely used culture media and growth conditions to recover a large fraction of the microbial population[185]. Culture-based studies are, therefore, nowadays used to a lesser extent. However, culture-based studies also enable phenotypic as well as genotypic identification, and especially in those cases where phenotypic identification is crucial (e.g. antibiotic resistance[186]), culture-based studies can still offer more information than purely genotypic studies.

1.3.3 Molecular techniques

A commonly used target for bacterial detection and enumeration is 16S rRNA. Ribosomes are two-subunit rRNA-protein complexes. In prokaryotes, the small subunit includes a 16S rRNA, whereas the large subunit includes 23S and 5S rRNAs. 16S rRNA sequences can be used to infer phylogenetic relationships, and to identify unknown microbes by database comparisons[187]. Since DNA remains relatively intact in non-viable cells, the methods targeting DNA (e.g. 16S rRNA gene) enumerate both viable and dead cells[188-189]. RNA is more labile than DNA and more susceptible to degradation caused directly by deleterious conditions/treatments[190]. Due to the fact that environmental conditions influence the cellular rRNA content, the amount of rRNA in cells is considered to correlate with the growth rate[191]. However, the rRNA molecule remains stable for some time after cell death; therefore, the rRNA content of a bacterium may not correctly reflect its physiological status. A more attractive molecular indicator molecule for bacterial viability would be messenger RNA (mRNA), due to its rapid degradation after cell death. However, the extent of degradation varies according to the type and severity of the treatment used for killing of the bacteria[190]. Furthermore, since detection of mRNA in viable cells may depend on their physiological status, cells that are stressed, or viable but poorly culturable, may contain too low quantities of mRNA for detection[192]. In addition to 16S rRNA, other housekeeping or functional genes – such as genes associated with sulfate

reduction (*dsr*AB gene) or methane (*mcr*A gene) or butyrate (butyryl-*Co*A:acetate *Co*A-transferase gene) production[193-196] – may be used as targets for PCR and/or hybridization. However, the sequence databases for other genes besides 16S rRNA contain only limited numbers of sequences, thus limiting their use in microbial ecological studies[188].

1.3.3.1 Hybridization

Hybridization techniques can be used in bacterial identification either alone or combined with a preceding PCR step. In hybridization, a labeled probe anneals to a denatured target DNA / RNA with sequence homology[197-198]. Target DNA / RNA can be directly blotted onto a membrane, or if size information of the hybridization target is warranted, the target DNA / RNA is first run through an agarose gel and then transferred to a membrane. Detection of hybrids is based on radioactive signal, fluorescence, or color reaction, depending on the type of label. By determining the intensity of the hybridization signal, the number of target organisms can be estimated[199]. However, it should be noted that the relative amount of an rRNA sequence does not reflect the true abundance of the microbe, since the amount of rRNA per cell varies according to the species and the metabolic activity of the bacterial cell[200]. Nevertheless, the relative quantity of rRNA provides a reasonable measure of the relative physiological activity of a specific population[201]. Dot-blot hybridization has been used to measure the quantity of a specific 16S rRNA in a mixture relative to the total amount of rRNA from human GI-samples in several studies[15,59,62,67,70,73,80,202-204].

Microarrays, which are miniaturized and automated forms of a dot-blot hybridization, facilitate the study of large numbers of genes simultaneously by hybridization of DNA or mRNA to a high-density array of immobilized probes[205-207]. There are two major types of DNA microarrays; an oligonucleotide-based array and a PCR product-based array. Currently there are several microarrays developed for the analysis of human oro-gastrointestinal tract microbiota[208-219].

The detection of whole bacterial cells via labeling of specific nucleic acids with fluorescently labeled oligonucleotide probes is called fluorescent *in situ* hybridization (FISH). FISH requires no cultivation and cells can be fixed before analysis, thus enabling the storage of samples prior to analysis[220-221]. The whole-cell or *in situ* hybridization technique has become a much used molecular tool in microbial ecology, since organisms or groups of organisms can be identified with minimal disturbance of their environment and spatial distribution. FISH in combination with epifluorescence microscopy and/or flow cytometry is a widely applied method to analyze and quantify microbial communities of the human oro-gastrointestinal tract[15-16,63-64,66-69,71,74,77,222-227].

Multiplexed quantification of bacterial 16S rRNA by solution hybridization with oligonucleotide probes and affinity capture (transcript analysis with the aid of affinity capture; TRAC) has also been used for the detection of GI microbiota[65,228]. In TRAC, biotinylated RNA is hybridized with multiple probes. Thereafter, the hybrids are affinity captured on streptavidin coated paramagnetic beads and washed. The hybridized probes are eluted and their quantity and identity are determined by capillary electrophoresis. The signal intensities of the recorded probes correspond to the amount of target nucleic acid in the mixture, while the size indicates the target[228].

1.3.3.2 PCR-amplification

Reliable and reproducible lysis of microbial cells, as well as the extraction of intact nucleic acids from any habitat is a demanding task[229-230]. In addition, removal of substances, which may interfere with hybridization or PCR amplification, may be difficult[229]. The cell lysis can be performed by enzymatic (e.g. lysozyme, lyticase or proteinase), chemical (e.g. detergents or guanidium isothiocyanate), or mechanical procedured (e.g. freeze-thaw/freeze-boil cycles, bead-beating, or microwave heating). In many cases, e.g. for the identification or fingerprinting of isolates obtained by culture, the crude cell lysate can be used directly in a subsequent molecular analysis. However, since oro-GIT samples contain inhibitory compounds, crude cell lysates are not sufficient when direct molecular detection methods are applied. Further processing steps include removal of proteins, which has commonly been performed by phenol-chloroform extraction, followed by precipitation of nucleic acids by ethanol, isopropanol or polyethylenglycol precipitation, and purification of nucleic acids[231-232]. Numerous articles have been published in which different DNA extraction protocols have been described for GIT samples[233-240]. When the metabolically active fraction of the community is of interest, the analysis should be performed with RNA rather than DNA. However, while extracting RNA from oro-GIT samples, special attention should be paid to avoiding degradation of RNAs with RNAses during the extraction procedure (for a review, see[230]). There are several articles describing different RNA extraction procedures for the GI-tract microbiota[239,241-243]. In addition, there are also several commercial kits available for the DNA and RNA extractions[237-238, 244-246]

In PCR, a thermostable DNA polymerase is used to exponentially amplify a target DNA sequence defined by two oligonucleotide primers[247]. The amplified DNA fragment can be visualized by agarose gel electrophoresis, which also allows the size determination of the PCR product, or by hybridizing the PCR product with a labeled probe. PCR techniques are very sensitive, and small amounts of contaminating DNA carried e.g. from one run to the next can give false positive results. Many types of sample matrices (e.g. feces) contain factors which can either totally inhibit the PCR reaction or cause partial inhibition leading to a non-exponential amplification of the target DNA. Inhibition may be avoided or reduced by pre-PCR sample manipulations such as dilution of the sample material, optimization of the used DNA/RNA extraction protocol, or by harvesting the bacterial cells from the sample e.g. by centrifugation, filtration, or using immunomagnetic beads coated with specific monoclonal antibodies to the target organism. However, even partial inhibition of the PCR reaction inevitably leads to reduced sensitivity and excludes the possibility of performing quantitative PCR. To minimize the risk of obtaining false-negative amplification results suitable external standards, which are co-amplified together with the target DNA in the PCR reaction, may be used[230,248].

When PCR is applied to human oro-GIT samples several problems arise including inhibition of PCR amplification by co-extracted contaminants, differential PCR amplification, formation of PCR artefacts e.g. chimeric molecules (leading to description of non-existing species), and DNA contamination. It should also be noted that 16S rRNA gene sequence variations due to ribosomal RNA operon heterogeneity can interfere with the analysis (for a review, see[230]). When PCR is used in direct bacterial detection from sample materials containing other microbes, the validation of the protocol applied is of utmost importance. The chosen method must be tested with a large panel of strains representing the target

species, closely related species, and other microbes commonly present in the sample material[249]. If only *in silico* testing is performed, the specificity of the applied PCR may not necessarily be as expected. This, and the fact that different methods must be applied to overcome the inhibitory effects of different sample matrices, necessitates the use of tailor-made approaches for each microbe-sample matrix pair. A positive control for each analysis is important in order to confirm that inhibitory substances do not interfere with the detection and cause false negative results.

Quantification of the initial amount of target is not possible in traditional end-point PCR because the amount of PCR product is already determined when the reaction has reached the plateau phase. In real-time PCR (qPCR), the amount of PCR product is measured at each cycle, including during the exponential phase, which enables quantification of the initial template amount. The real-time measurement is based on fluorescent dyes that either bind to double strand DNA or hybridize to a specific sequence. Since q PCR is especially vulnerable to inhibitory compounds, internal standards should always be used when complex sample matrices are studied[248]

There are numerous articles in which PCR and/or qPCR have been applied to samples from the human gastrointestinal tract[145,236-237,250-255]. However, nowadays endpoint PCR is mainly used as a preceding step before further characterization with DGGE, RAPD, microarrays etc.

1.3.3.3 Community analysis

Genetic fingerprinting techniques can be used to characterize bacterial communities or single bacterial isolates (Section 1.3.3.4). Genetic fingerprinting techniques of microbial communities provide a pattern or a profile of the community diversity based on the physical separation of unique nucleic acid sequences. Community fingerprinting techniques are relatively easy and rapid to perform and they allow simultaneous analysis of multiple samples, enabling the comparison of genetic diversities of microbial communities from different habitats, or studying the behavior of individual communities over time. Denaturing gradient gel electrophoresis (DGGE) has been used in numerous studies for characterization of the microbiota within oral cavity, colon, and feces[56-57,65,237,242,256-260]. Temperature gradient gel electrophoresis (TGGE), single-stranded conformation polymorphism (SSCP), and terminal restriction fragment length polymorphism (T-RFLP) have been used to a lesser extent than DGGE in the characterization of the human GIT[45,48,56,261-264]. In DGGE and TGGE, PCR-amplified DNA fragments of the same length but with different DNA sequences can be differentiated. Separation in DNA fragments is based on the electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide; DGGE) or a linear temperature gradient (TGGE). Partially melted DNA fragments are held together with a G+C-rich oligonucleotide, a GC-clamp. Therefore, each denaturing fragment theoretically generates only a single band in the gel. DGGE analysis combines a direct visualization of bacterial diversity and the opportunity for subsequent identification of community members by DNA fragment sequence analysis or hybridization with specific probes. However, DGGE has some specific limitations. DGGE can be used to separate only relatively small fragments, and it displays only the rDNA amplicons obtained from the predominant (over 1% of the population) species present in the com-

munity[265-266]. The presence of heterogeneous 16S rRNA genes (16S rRNA genes that exhibit small sequence variations in the genome of a given strain) can result in several bands in a DGGE/TGGE profile[56,256,267]. Furthermore, a single band may represent more than one strain[242,256,268]. The construction of 16S rRNA gene clone libraries and the screening for different clones by DGGE may overcome these deficiencies[269-270].

Denaturing high-performance liquid chromatography (DHPLC) has also recently been utilized in microbial community fingerprinting[271-272]. In DHPLC, PCR-products are separated using HPLC equipment instead of a denaturing gel, which enables automation and higher throughput than in manually handled DGGE. DHPLC has already been used for the analysis of human fecal samples[271,273-274], in which the new method proved to be as efficient as DGGE. However, the microbiota in the samples has not been as diverse as e.g. the predominant microbiota of elderly subjects. A drawback of the currently used DHPLC-method is the price of the equipment. However, a recent study demonstrated that traditional HPLC equipment may also be used for the analysis[275].

Clone libraries have been applied in gut microbiota research to a great extent. After the PCR step, the amplification product is purified, ligated to a vector and thereafter the ligated products are transformed into competent *E. coli* cells. The colonies of ampicillin-resistant transformants are randomly picked and thereafter processed for sequencing[61]. The first clone libraries constructed from the human gut microbiota were sequenced using Sanger sequencing[55,61,276-277], whereas in recent publications next-generation sequencing (NGS) has been applied for direct sequencing of the DNA-template (see Section 1.3.3.5 Metagenomics) or for sequencing of the PCR-products (e.g.[41,278-289]). In addition, in most of the Human Microbiome Project publications (http://hmpdacc.org/pubs/publications.php) NGS has been applied. There are several different approaches to NGS, depending on the equipment manufacturer, which were nicely reviewed by e.g. Shendure & Ji[290], Metzker[291], and Kuczynski *et al.*[292]. A common feature of all the NGS techniques today is that a massive amount of data is generated rather rapidly and with rather low cost. However, since the amount of data is indeed massive, the bioinformatics-part of the data is a demanding and laborious step, thereby counteracting the cost-effective approach.

1.3.3.4 Typing of bacterial isolates

Fingerprinting of bacterial isolates can be performed by a variety of techniques including e.g. ribotyping, amplified ribosomal DNA restriction analysis (ARDRA), pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD), repetitive element sequence-based-PCR (rep-PCR), and amplified fragment length polymorphism (AFLP). All these techniques aim at differentiating bacterial isolates at the subspecies level, preferably even at the strain level. PFGE and RAPD were used in the experimental part, and they are therefore discussed in more detail.

Random amplified polymorphic DNA (RAPD). In RAPD fingerprinting, one or two primers (usually 10–12 bp long) are arbitrarily selected and allowed to anneal to the bacterial genomic DNA template at a low stringency, thus resulting in several amplification products of varying sizes. The products are thereafter resolved electrophoretically to yield a RAPD-fingerprint[293]. RAPD typing is fast to perform, especially if the fingerprinting can be performed directly on single colonies growing on an agar plate. Due to the low stringency of the PCR amplification, RAPD-fingerprints can show some variation (especially in band strength)

and therefore the fingerprint comparisons must be performed visually by an experienced person. However, when strictly identical conditions (same thermocycler, reagents etc.) are used, the method is usually reliable[294]. RAPD banding pattern reproducibility can be improved by using a procedure in which the same strains are exposed to three different annealing temperatures (with increasing stringency) and by identifying the stable amplicons[295]. This triplicate procedure naturally makes the RAPD fingerprinting technique more laborious. RAPD is best suited for studies in which a specific bacterial strain (e.g. a certain food-borne pathogen) is searched among a large number of isolates. With RAPD, bacterial isolates with clearly different fingerprints can be quickly identified and rejected and the remaining – fewer – strains then further characterized with another, more laborious technique (e.g. ribotyping, PFGE, AFLP). RAPD is not well suited for inter-laboratory or taxonomical studies or for studies in which the aim is to develop a fingerprint database.

Pulsed-field gel electrophoresis (PFGE). Due to the problems encountered with conventional ribotyping of bacterial genomes, bacterial fingerprinting technique was developed in which profiles consisting of fewer numbers of larger sized genomic restriction fragments were obtained. In this technique, bacterial genomic DNA is restricted *in situ* (in a gel block) with a rare cutting restriction endonuclease such as SmaI, SfiI, NotI, or BssHII, and the restriction fragments are separated by pulsed-field gel electrophoresis (PFGE; a special technique capable of resolution of large DNA fragments)[296]. With PFGE highly discriminative fingerprinting of bacterial isolates can be performed. Of the different molecular fingerprinting methods PFGE has in many cases proved to be the most discriminative[142]. However, PFGE is a laborious technique and it is not usually applied in studies in which large numbers of isolates are characterized.

1.3.3.5 Metagenomic studies

Metagenomics may be defined as the application of NGS to a DNA-template obtained directly from a sample[142]. Recently there have been several metagenomic studies on the human gut microbiota e.g.[4,54,298-305]. In most studies, metagenomic approach is applied to study the genes present in the microbiota. The gene-pool of human fecal microbiota has been found to be enriched with genes responsible for e.g. (pro)phage-related proteins, biodegradation of complex sugars and glycans, carbohydrate fermentation (e.g. mannose, fructose, cellulose, and sucrose), vitamin production etc.[2,4,54], which is not surprising taking into account the known properties of the most abundant human gut microbes (Section 1.2 of this thesis). As with any NGS-technique, the amount of data obtained is enormous. However, metagenomic studies are usually performed with Illumina technique, and thus the obtained sequence length is usually <200 bp. This makes the bioinformatics part even more demanding than when NGS is applied to a PCR-product.

1.3.4 The effect of the used method on the results

Most of the steps in the processing of fecal samples (Figure 5) may affect the microbiological results obtained. The sampling and storage conditions have been shown to affect the results[280,306-307], although not as extensively as the following steps, especially the DNA extraction[236-237,308]. The initial two week storage at -20^{o}C has been shown to decrease the

detected numbers of *Bacteroides* spp. by over 1 log unit as compared to the fresh samples, whereas the storage temperature has not been shown to have such a significant effect on the gram-positive bacteria (e.g. Firmicutes and Actinobacteria)[237]. Since the analysis of fresh fecal samples, especially in the case of large cohorts, is impractical and mostly also impossible, the effect of the storage conditions on the results obtained should be taken into account when the results are discussed. When DNA-extraction is performed using solely chemical treatments combined with heat treatments, it has been shown that the results for some bacterial groups may be as much as 4 log-values lower as compared to a DNA extraction method in which rigorous mechanical disruption is applied[237].

Actinobacteria, in particular, have been shown to be underestimated with many currently used techniques, most probably due to their high G+C content[309]. In a study of Nakamura *et al.*[310], it was shown that when the same samples were examined with qPCR (enzymatic DNA-extraction) and FISH, the difference in results was enormous. The proportions of bifidobacteria with qPCR was detected to be 0.1 -1.7%, whereas the proportion with FISH was 28–84%[310]. Moreover, in studies in which clone libraries have been constructed, the primer Bact-8F (synonyms: e.g. 008F) targets bifidobacteria only poorly[262], and therefore in studies in which this primer has been used the abundance of bifidobacteria has been underestimated (e.g.[55,61,311]). In addition, the normally used 95^{o}C for denaturation in PCR-amplification may not be sufficient for high G + C content bacteria, such as Actinobacteria and SRB[312].

Another methodological aspect that may cause confusion is that different probes/primers target different bacterial populations, such as in the detection of Bacteroidetes and Firmicutes. In pyrosequencing/metagenomic studies the authors usually refer to Bacteroidetes, whereas in studies conducted with FISH/PCR, the studied bacterial population is usually narrower (e.g. *Bacteroides* spp. or *Bacteroides-Prevotella* group)[313]. The same controversy applies to Firmicutes. In many pyrosequencing studies Firmicutes are discussed as a single group, whereas in fact it contains several different bacterial classes – e.g. Bacilli, Clostridia, Erysipelotrichia – which all have different metabolic properties. Furthermore, when PCR/FISH is used, each of these groups is usually studied separately. Moreover, since the phylum Bacteroidetes is more thoroughly characterized than the phylum Firmicutes, it is possible that the representation of Bacteroidetes in metagenomic studies is over-estimated.

When microbial communities are examined, especially using several different techniques and/or next-generation sequencing, the quantity of data generated is enormous. Therefore, the use of suitable algorithms for e.g. measurement of microbial diversity and correct interpretation of the results are of utmost importance[292,314-320]. Moreover, we should also bear in mind that even if statistical significance is achieved, the observed changes may not be of biological significance.

1.4 Factors affecting the autochthonous microbiota

There are several factors that affect the composition of the human gastrointestinal microbiota, and thus potential dysbiosis (Figure 8).

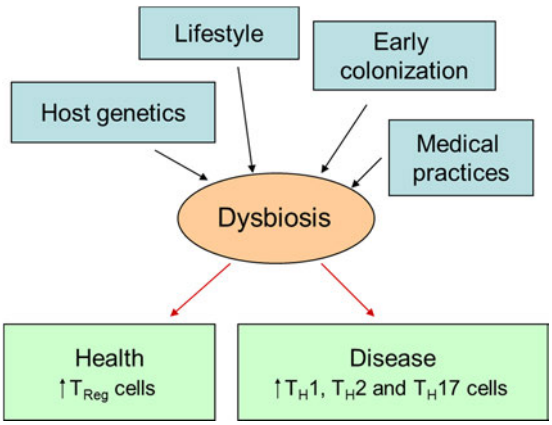

Figure 8. Proposed causes of dysbiosis of the microbiota (adapted from[292, 314-320])

1.4.1 Age

Bacterial colonization of the infant gastrointestinal tract is influenced by e.g. mode of delivery, prematurity, type of feeding (breast feeding vs. formula feeding), antibiotic treatment of the child or the mother, lifestyle, and geographics[225-226,274,321-324]. The earliest colonizers are usually facultative anaerobic bacteria such as Enterobacteriaceae, streptococci and staphylococci, whereas later colonizers tend to be strict anaerobes e.g. bifidobacteria, clostridia, and *Bacteroides* spp. regardless of the infant's geographical origin and methods used for the detection[225,270,311,325-328]. Immediately after birth, within the first 24-h, the rectal microbiota of vaginally delivered babies has been shown to resemble their own mother's vaginal microbiota (i.e. dominated by *Lactobacillus*, *Prevotella*, or *Snethia* spp.), whereas the rectal microbiota of babies delivered by Caesarean section (C-section) resembled that of the skin (e.g. *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp.)[329]. Infants delivered by C-section harbor fewer bifidobacteria and higher proportions of *Bacteroides*, the *Atopobium* cluster, and *C. difficile* at early infancy than those delivered vaginally. In addition, at the age of one month, the total gut bacterial count of vaginally delivered infants has been reported to be higher as compared to infants delivered by C-section[225,330]. The gut microbiota of preterm infants is less diverse than those of full-term babies, and the main bacterial groups found have been *Enterococcus*, *Streptococcus*, *Staphylococcus*, and *Clostridium* spp., whereas the levels of *Bifidobacterium*, *Bacteroides*, lactobacilli, and the *Atopobium*-cluster are lower[262,274,323,331-332].

There are numerous studies in which the predominance of bifidobacteria in exclusively breast-fed infants has been found (regardless of the detection method used)[143,225, 255, 324, 333-335]. At the age of 3–6 weeks, exclusively breast fed infants harbor higher numbers of bifidobacteria, whereas formula-fed babies have more diverse microbiota, lower numbers of bifidobacteria and higher numbers of *Bacteroides*, *Eubacterium rectale* group, *Lactobacillus* group, *C. difficile*, and the *Atopobium* cluster[225,321, 324,333,336-337]. At the age of two years, the breast-fed children have been reported to have higher numbers of enterococci[322].

Children who have received antibiotics during the first year/s of life have lower numbers of enterococci and lactic acid bacteria as detected by culture-based analysis, and *Bac-*

teroides and bifidobacteria as detected with molecular techniques[321-322]. Lifestyle has also been shown to be associated with the development of the gut microbiota. Children living according to an anthroposophic lifestyle (restricted use of antibiotics, greater consumption of vegetables, etc.) have been reported to have higher bacterial diversity than omnivorous control children and farm children (greater consumption of farm milk, contact with animals, etc.). The farm children were reported to have the lowest bacterial diversity as compared to the children living according to anthroposophic lifestyle and control children[261].

In addition to the aforementioned factors affecting the intrinsic microbiota of infants, mother's weight at delivery and weight-gain during the pregnancy, have also been associated with the infants' microbiota. Fecal *Bacteroides* and *Staphylococcus* were higher in infants of over-weight mothers during the first 6 months of life. Furthermore, prevalence of *A. muciniphila* and *C. difficile* were lower in infants of normal-weight mothers and of mothers with normal weight gain during pregnancy[338]. However, the microbiota of the mothers was not studied. Longer daily time of crying and fussing in infants of 7–12 weeks of age has also been associated with lower numbers of bifidobacteria and lactobacilli[339].

By the end of the first year of life, when the child has already started to eat the same foods as the adults, the gut microbiota starts to converge towards a profile characteristic of the adult microbiota[311,337]. However, the fecal bacterial diversity is still lower[312]. By the end of the second to third year, the phylogenetic composition evolves towards the adult-like composition[335,340].

It was already shown several decades ago that the gastrointestinal microbiota evolves with age[341]. Dental deterioration, salivary function, digestion, slower intestinal transit time, and changes in diet and physical activity may affect the gastrointestinal microbiota of aging people. Interest, as well as the number of studies, in the gastrointestinal microbiota of elderly people has grown as life expectancy in the western world has rapidly increased. The elderly have been reported to have relatively stable microbiota[211,278,342]. However, the microbiota of the elderly has been reported to be more diverse and to contain partly different core microbiota as compared to younger adults[211,278,343-344]. Moreover, inter-individual variation is greater in elderly people as compared to younger adults[14,278,344-345].

In culture-based studies, Asian elderly subjects have been reported to have lower levels of bifidobacteria and higher levels of clostridia, lactobacilli, streptococci, and Enterobacteriaceae than younger adults[346-347]. Moreover, in European culture-based and molecular studies the results have been partly contradictory[64,204,211, 278,342,345,348-350]. Moreover, the difference in several bacterial groups between elderly people and younger adults has been shown to be dependent on geographical location[64]. The number of bifidobacteria has been shown to be lower in most of the European studies in elderly subjects as compared to younger adults[64,204,278,342,349-353]. The absence of bifidobacteria, or their low numbers in the elderly, may have metabolic and health consequences for the host, since they affect immune system function and a multiplicity of other functions – e.g. synthesis of vitamins and protein, and supplementation in digestion and absorption[204,347]. Furthermore, genomic DNA of some bifidobacterial strains has been shown to stimulate production of the Th1 and pro-inflammatory cytokines, interferon (IFN)-gamma and TNF-alpha[354]. In addition, bifidobacteria are involved in colonization resistance in the bowel[204]. Besides reduction in numbers, diversity of the bifidobacterial population has been reported to decrease with age in some studies[342,351], whereas others have had contradictory results[344].

The abundance of the *Bacteroides – Prevotella* group has been reported to decrease in French, Italian, Swedish, Dutch, and Finnish elderly people, whereas in German and Austrian elderly people an increase was observed with molecular techniques[64,211,255,348]. By contrast, German elderly people were reported to have decreased numbers of *Bacteroides – Prevotella* group bacteria using culture-based techniques[355] and Finnish elderly people had a higher relative proportion of *Bacteroides* spp. with clone library based analysis[356]. Moreover, in recent pyrosequencing-based studies of Irish elderly people, it was reported that Bacteroidetes was the most dominant fecal bacterial phylum in 68% of the elderly individuals, with an average proportion of 57% (range 3% to 92%). More than half of the core microbiota (53%) in the elderly subjects comprised Bacteroidetes belonging to the genera *Bacteroides* (29%), *Alistipes* (17%), and *Parabacteroides* (7%) as compared to 8–27% in the younger adults[278,345].

In the Irish elderly people the phylum Firmicutes had an average proportion of 40% (range 7% to 94%), whereas proportions of the total number of reads for clusters IV and XIVa ranged from 1.7% to 34% and 1.1% to 22%, respectively[278,345]. The abundance of bacteria belonging to the Clostridial cluster XIV has been reported to clearly increase in the German elderly population, whereas a clear reduction has been observed in the Italian, Dutch, Finnish, and Japanese elderly populations[64,348,356-357]. In addition, significant increase in Clostridial cluster XIV diversity and reduction in relative abundance of *Eubacterium hallii*-like bacteria has been observed in Finnish elderly people[211,344].

F. prausnitzii subgroup and Clostridial cluster IV have been reported to clearly decrease in Italian, Swedish, Dutch, French, and Austrian elderly people[64,348,352-353], whereas in Irish elderly people the proportion of *Faecalibacterium* spp. and consequently Clostridial cluster IV was higher than in younger adults[278]. Similarly to these studies, copy numbers of the butyryl CoA:acetate CoA-transferase gene responsible for butyrate production have been shown to be reduced in elderly Austrian people[358].

In many studies the abundance of lactobacilli has been shown to increase or to remain stable, regardless of the nationality of the studied elderly people[211,255,347-348,352,355-356]. The levels of enterobacteria have been reported to increase with age in German, Dutch, and French elderly populations[255,348,355], similarly to the studies of Asian elderly people[357].

Italian centenaries have been reported to have less diverse microbiota than the elderly (aged 63 to 76 years; average 73) or the younger adults (aged 25 to 40 years; average 31). The microbiota of the centenaries was enriched in facultative anaerobes, mostly belonging to Proteobacteria and Bacilli. In addition, the centenaries tended to have a higher prevalence of Archaea. On the other hand, the relative proportion of *E. rectale* group was lower in the centenaries than in the elderly and in the younger adults and numbers of bifidobacteria were lower. Furthermore, although the relative proportion of the *C. leptum* group was unaffected, the proportion of the *F. prausnitzii* subgroup decreased, indicating rearrangement in the species composition of the *C. leptum* group[359].

1.4.2 Gender

There are not many articles in which the effect of gender on the gut microbiota has been studied, although some differences have been found; e.g. the numbers of the *Bacteroides-Prevotella* group in feces have been found to be higher in males than in females[64,360]. Most likely there are also other not yet reported differences, since there are

many anatomical, hormonal, and other physiological differences between the genders, but due to other confounding factors bacterial differences between genders are not easily detectable. Moreover, the microbiota of premenopausal and postmenopausal healthy women has been shown to differ, stressing even more the hormonal effects[361].

1.4.3 Genetics of the host and geography

Twins, especially monozygotic (MZ) twins, have been reported to have more similar interindividual fecal microbiota than unrelated people. In addition, twins and their mothers have been found to have a more similar microbiota than unrelated individuals. These findings have led to the conclusion that the host genotype affects the development of the gut microbiota and gut bacterial composition[57,83,284,362-363]. However, the aforementioned studies have been conducted with MZ pairs concordant for leanness or obesity. Simões *et al.*[364] studied MZ twins concordant and discordant for obesity, and found that the concordant normal-weight MZ twins had more similar bacterial populations than the MZ twins discordant for obesity. Especially those discordant twins whose liver-fat values differed greatly had less similar bacterial populations. These findings also address the importance of the diet in addition to the genetic drivers[364]. Although the genetics or the shared environmental factors during upbringing result in more similar bacterial populations, viromes have been shown to be unique to individuals regardless of their degree of genetic relatedness[79].

Besides genetics, the effect of the geographic origin (which may also include genetic differences) has also been studied in relation to the gut microbiota composition. Even in Europe some differences may be found between different countries, e.g. proportions of bifidobacteria have been found to be two- to threefold higher in an Italian adult study population than in other European study populations. Other bacterial differences, although non-significant, were also found in the European studies[63-64]. In addition, when 6-week old infants across Europe were studied, geography was found to be a more prominent factor than delivery mode, breast-feeding, and antibiotics. In infants, children from Northern European countries had more bifidobacteria, whereas the southern European infants were associated with more diverse microbiota and higher numbers of *Bacteroides* than the Northern European children[225]. In the studies involving people from different continents, the bacterial population differences have been more distinct. In a comparison of healthy people from southern India and the United States, it was found that 36% of the Americans as compared to 14% of the Indians were able to metabolize digoxin (metabolized by e.g. *Eggerthella lenta*)[365]. Furthermore, in a few recent studies there have been consistent results that the European and North-American fecal microbiota differs significantly from the Southern-American, African and Chinese fecal microbiota[335,360,366]. However, in all these studies it is not possible to exclude the impact of other possible confounding factors, and therefore dietary habits and genetics may also contribute to the differences.

1.4.4 Diet

The main external factors that can affect the composition of the microbial community in generally healthy adults include major dietary changes and antibiotic therapy. The effect of diet on the GI-tract microbiota is not completely clear, although changes in some se-

lected bacterial groups have been observed due to controlled changes to the normal diet – e.g. high-protein diet [367-368], prebiotics[344, 369-371], probiotics[372-377], weight-loss diet[82,378-379] and berries[380]. More specifically, changes in the type and quantity of non-digestible carbohydrates in the human diet influence both the metabolic products formed in the lower regions of the GI-tract and levels of bacterial populations detected in feces[381]. The interactions between dietary factors, gut microbiota and host metabolism are increasingly demonstrated to be important for maintaining homeostasis and health[382].

Diet influences bacterial metabolism, and specific items in the diet may have selective effects in the microbiota, which may be important for health[51]. It has been shown with culture-based methods[367] that the counts of *Bacteroides* sp., *B. vulgatus*, *B. fragilis*, and unidentified clostridia increased significantly, whereas counts of *Bifidobacterium adolescentis* decreased significantly during a high-beef diet as compared to a meatless diet (fat and fiber contents were essentially the same in both diets). In addition, sulfide concentrations have been shown to be high on a high-beef diet[383]. Since H_2S is toxic to the colonic epithelium and sulfide has been shown to inhibit butyrate oxidation, dietary sulfide may selectively stimulate the growth of a single group of bacteria, namely SRB, with potentially harmful effects on the epithelium[51,384]. In addition, diet with high protein and low carbohydrate content has been shown to reduce the numbers of *Roseburia* / *E. rectale* group, while increasing proportions of branched-chain fatty acids and concentrations of phenylacetic acid and *N*-nitroso compounds[368]. Moreover, it should be noted that the World Cancer Research Fund released in May 2011 a report based on 1012 clinical trials, in which red and processed meat were convincingly associated with increased risk, whereas foods containing dietary fiber, in particular cereal fiber and whole grains, were associated with decreased risk of colorectal cancer[385-387].

The impact of habitual diet on fecal microbiota has been studied for decades. In older culture-based studies it was found that numbers of bacteroides were lower and numbers of enterococci and *E. coli* higher in Ugandan, Indian, and Japanese people on high-carbohydrate diet as compared to people on a Western diet[388-389]. However, when English people on a strictly vegetarian diet were studied, their microbiota resembled those of people on a Western diet more than the microbiota of other vegetarian people from different continents[388]. Similarly, numbers of bacteroides and clostridia were lower in the Nigerian Maguzawa tribal people (predominantly cereal diet) than in the other dietary groups (cereal with regular meat consumption and cereal with frequent consumption of cows' milk)[390]. In more recent molecular studies, in which gut microbiota from different parts of the world has been compared, a significant correlation between habitual diet and fecal microbiota has also been found. African children consuming a diet low in fat and animal protein and rich in starch, fiber, and plant polysaccharides (predominantly vegetarian) had significantly more Bacteroidetes and Actinobacteria and less Firmicutes and Proteobacteria than European children, who had a diet high in animal protein, fat, sugar, and starch, but low in fiber. Moreover, members of the genera *Prevotella* and *Xylanibacter* were found exclusively from the African children[366]. Partly similarly to these findings, *Prevotella* enterotype was found to be associated with high consumption of carbohydrates, whereas *Bacteroides* enterotype was associated with high consumption of animal protein, amino acids, and saturated fats[391]. Strict vegetarians have been shown to have higher percentages and/or numbers of *E. rectale – B. coccoides* group and *C. ramosum* group (Clostridial cluster XVIII) bacteria than omnivorous people[264, 358], whereas vegans have been shown to have lower fecal numbers of

*Bacteroide*s spp., *Bifidobacterium* spp., *E. coli*, and Enterobacteriaceae[392]. In addition, both vegetarian and vegan habitual diets have been associated with the *Prevotella* enterotype[391]. In older culture-based studies, no fusobacteria were found in fecal samples of vegetarian people, whereas the mean count in both Japanese and America fecal samples the levels were over 10^8 cfu/g. Furthermore, the total count of anaerobic streptococci, *Peptostreptococcus* spp., *Actinomyces* spp., and *Lactobacillus* spp. were higher in strict vegetarians as compared to people on a traditional Western diet[14,389,393-394].

1.4.4.1 Dietary fiber

Dietary components that escape digestion by endogenous enzymes in the upper gastrointestinal tract become available substrates in the large intestine[6]. Dietary fiber (DF) is a normal constituent of most foods derived from plants[395]. These “non-digestible” (ND) dietary carbohydrate substrates include resistant starch, plant cell wall material (non-starch polysaccharides), and oligosaccharides[6]. In the human colon, the DF is metabolized by the microbiota to short chain fatty acids (SCFA), comprising mainly acetic, propionic, and butyric acids. SCFA have been implicated to have both local and systemic beneficial biological effects in the human body; acetate is readily absorbed and transported to the liver; propionate is a substrate for hepatic gluconeogenesis; and butyrate is the preferred fuel of the colonocytes and also plays a major role in the regulation of cell proliferation and differentiation[6].

In vitro and in vivo evidence indicates that a group related to *F. prausnitzii*, *Roseburia,* and *E. rectale* plays a major role in mediating the butyrogenic effect of fermentable dietary carbohydrates[112,223,396-397]. In addition, it has been shown in numerous studies that as dietary carbohydrate content is reduced in the diet the count of *F. prausnitzii* declines respectively[381,398]. However, some lactate-utilizing bacteria of the Clostridial cluster XIV produce less butyrate in the presence of lactate-utilizing SRB. Moreover, in the presence of higher abundance of lactate, the formation of butyrate was reduced even more and the formation of hydrogen sulfide was promoted[399].

Cereal grains are a good source of DF. The main DF components of cereal grains are arabinoxylan (AX), cellulose, β-glucan, fructan, resistant starch, and lignin[400]. The gut microbiota-stimulating activities of AX (stimulates *Bacteroides* spp. and *Roseburia* spp.), resistant starch (stimulates bifidobacteria, *Bacteroides* spp., *Ruminococcus bromii*, *E. rectale*, and *Roseburia* spp.), β-glucan (stimulates bifidobacteria), and fructan (stimulates bifidobacteria, *Bacteroides* spp., lactobacilli and butyrate-producers) are well recognized[401-409]. In addition, arabinoxylan-oligosaccharides (AXOS), which are enzymatic hydrolysis products of AX, have been shown to stimulate the growth of bifidobacteria in some studies[404]. The effects of cellulose and lignin on gut microbiota are less well known [410-411]. However, it should be noted that e.g. the size of the grain flakes may lead to different bacterial responses: i.e. it has been shown that smaller sized whole oat grain flakes (0.53–0.63 mm) resulted in a significant increase in the numbers of *Bacteroides – Prevotella* group bacteria, whereas in a fermentation with larger oat flakes (0.85–1.00 mm) bifidobacterial numbers increased[412]. It has been shown in *in vitro* model studies using different substrates that the majority of the bacteria attached to wheat bran belonged to the Clostridial cluster XIV and some bacteria were *Bacteroides* spp., whereas *Ruminococcus bromii*, *Bifidobacterium adolescentis*, *Bifidobacterium breve*, and *E. rectale* were found attached to starch.

When mucin was used as a substrate, the most commonly found bacteria were *Bifidobacterium bifidum* and an uncultured relative of *Ruminococcus lactaris*[413].

1.4.4.2 Probiotics and prebiotics

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host, according to the widely accepted definition by FAO/WHO[414]. Most of the currently used probiotics belong to the genera *Bifidobacterium* and *Lactobacillus*. However, probiotic preparations containing species of the genera *Enterococcus*, *Pediococcus*, *Streptococcus*, *Lactococcus*, *Propionibacterium, Bacillus*, and *Saccharomyces* are also used[415]. The past two decades have seen a marked increase in the inclusion of probiotic bacteria in various types of food products, especially in fermented milks[416]. During recent years probiotics have also been increasingly incorporated into non-dairy foods such as fruit and berry juices and e.g. cereals[415]. In good quality products, the daily dose should be approximately 10^9 cfu/d[417]. Probiotics do not usually colonize the GI-tract, and therefore the products should be consumed daily for the health benefits[418]. In most of the studies, probiotics have not caused any significant changes in the predominant fecal microbiota of healthy adults. However, there are a few studies in which e.g. *L. rhamnosus* GG has been shown to modulate the fecal microbiota and increase overall bacterial diversity in infants[373,419]. In addition, *Bifidobacterium animalis* subsp. *lactis* Bb12 has been shown to reduce the numbers of Enterobacteriaceae and *Clostridium* spp. in preterm infants[374].

Prebiotics were originally described as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth, activity, or both of one or a limited number of bacterial species already resident in the colon[420]. The updated description of prebiotics is as follows; prebiotics are non-digestible (by the host) food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract. The prebiotics that currently fulfill the prebiotic criteria are inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and lactulose[421]. The best sources of naturally occurring prebiotics may be found in vegetables such as artichokes, onions, chicory, garlic, and leeks[418]. There are numerous studies in which the bifidogenic properties of prebiotics have been shown[239,288,370-371,421-425]. In addition, increase in abundance of lactobacilli[370-371] and *F. prausnitzii*[195,424] has been shown. Moreover, in infant formulas, GOS + FOS supplementation of cow's milk based formula has led to a bifidobacterial population, which resembled more that of breast-fed infants than purely formula-fed infants[426].

1.4.4.3 Medication

In addition to dietary components, the other important external factor affecting the microbiota is **antibiotic** consumption. However, different types of antibiotics have different types of action mechanisms and thus different effects on the gut microbiota[427-428]. In addition, individual human responses may be different. Bifidobacteria are typically susceptible to the majority of clinically relevant antibiotics such as penicillins, cephalosporins, and macrolides[429-434], and most of the commensal gut bacteria (e.g. bifidobacteria, *Bacteroides* spp.) to amoxicillin and clavulanate[430].

Penicillins are the most commonly used antibiotics among outpatients in EU[435]. In culture-based studies, which can only give a limited view of the microbiota, a typical finding in short-term studies in adults consuming amoxicillin has been an increase in the numbers of Enterobacteriaceae and *Klebsiella* spp., and reduction in bifidobacteria and bacteroides[428,436]. It has also been shown that amoxicillin consumption results in a significant reduction in the metabolic activity of gut microbiota (seen as reduced production of SCFAs)[376] and a major shift in the fecal dominant microbiota[437]. On the other hand, studies on the effect of amoxicillin on the gut microbiota in young children are limited. In a short-term (3 weeks) study performed in Chile, amoxicillin consumption resulted in a decrease in the total number of fecal bacteria (groups were not identified) and in an increase in the numbers of *E. coli*[438]. In addition, amoxicillin has been shown to alter the bifidobacterial population of infants; *B. adolescentis* disappeared and the occurrence of *B. bifidum* decreased[439].

Most cephalosporin administrations have been associated with increase of enterococci and significant colonization with *C. difficile*, as detected with culture-based methods. In addition, cefixime has also been shown to significantly suppress anaerobic microbes[436]. However, ceftaroline, a new broad-spectrum cephalosporin, did not significantly affect any studied fecal bacterial groups[440].

Oral quinolones have been shown to eliminate or strongly suppress bacteria within Enterobacteriaceae using a culture-based approach[436]. Ciprofloxacin (a member of the quinolone-family) ingestion has been shown to affect numerous fecal bacterial operational taxonomic units (OTU); the relative abundance of 43 OTUs decreased and 11 OTUs increased. Within the OTUs showing a decrease were eight OTUs related to *F. prausnitzii* and similar numbers of OTUs related to other Ruminococcaceae and Lachnospiraceae, seven OTUs within the genus *Bacteroides*, four in the genus *Alistipes*, and two in Porphyromonadaceae. The OTUs whose relative abundance increased during the ciprofloxacin ingestion also included OTUs within Lachnospiraceae and *Bacteroides* spp. Interestingly, only below 0.l% of the reads belonged to the Phylum Actinobacteria, which may be the reason why no difference in the Actinobacterial population was detected[441]. Ciprofloxacin + clindamycin treatment has also been reported to simplify the predominant microbiota[442].

Antibiotic use in elderly people has been reported to cause significant increase in the relative abundance of Bacteroidetes, especially *Parabacteroides* spp., and a decrease in the relative abundance of Firmicutes, Proteobacteria, and Actinobacteria. In addition, a low (250-mg) dose of clarithromycin decreased the proportion of Bacteroidetes (*Bacteroides* spp. and *Parabacteroides* spp.) and equivalently increased the proportion of Firmicutes, whereas a higher (500-mg) dose of clarithromycin increased the proportion of Bacteroidetes (*Parabacteroides* spp.) and non-equivalently decreased the proportion of Firmicutes[278]. The combination of clarithromycin and metronidazole (used for treatment of *H. pylori*) has been shown to cause a significant decline in bacterial species diversity of fecal microbiota and reduction in the relative abundance of Actinobacteria[443]. Clindamycin administration has been shown to cause a sharp decline in the clonal diversity (as detected with repetitive sequence-based PCR) of fecal *Bacteroides* spp. In addition, the fecal *Bacteroides* population never returned to its original composition during the two year study period[444]. Moreover, distinctly lower numbers of anaerobic bacteria have been observed after clindamycin administration[445-446].

Doxycycline has not been shown to cause major disturbances in the predominant culturable anaerobic microbiota. However, the numbers of streptococci, enterococci, entero-

bacteria, and fusobacteria declined during doxycycline intake[445-448]. In addition, doxycycline therapy has been shown to reduce fecal bifidobacterial diversity[449]. In the study of Borglund *et al.*[450], tetracycline significantly suppressed the culturable intestinal anaerobic microbiota, as well as streptococci and enterococci, and resulted in colonization by new tetracycline-resistant bacteria in several subjects. However, the effects of tetracycline antibiotics are quite significant on the aerobically growing intestinal microbiota; especially an increase of resistance in *E. coli* has been noted[451-453].

In many studies the antibiotic group administered has not been reported, and therefore the results of some studies cannot be grouped according to the relevant antibiotic. However, in several studies on children and infants a decrease in bifidobacteria and *Bacteroides* has been observed[321,454-455]. In elderly hospitalized people, a reduction in the prevalence of *Bacteroides – Prevotella* group, *Bifidobacterium* spp., *Desulfovibrio* spp., *Clostridium butyricum*, and *Ruminococcus albus* and an increase of *Enterococcus faecalis* have been reported[342,456].

The predominant fecal microbiota has been shown to return (as detected with TGGE) to a rather similar profile as before the antibiotic intake within a month[437]. Similarly, in more detailed pyrosequencing studies it has been shown that the majority of the fecal microbiota is restored in four weeks after the antibiotic treatment ended. However, several taxa (OTUs related to *Clostridium piliforme/Clostridium colinum* and *Bilophila wadsworthia*) were not restored even after a six month follow-up period[457]. Furthermore, in a pyrosequencing and T-RLFP based study of Jakobsson *et al.* it took four years post treatment for the fecal microbiota of some individuals to recover[443].

Proton pump inhibitors (PPIs) are commonly used to treat acid-related diseases of the stomach. Due to increased availability, many patients suffering from mild gastroesophageal reflux disease are nowadays relying on self-diagnosis and treatment. PPIs are designed to shut down the gastric proton pump of parietal cells, resulting in increase in the pH of the stomach[42]. Several studies have shown bacteriostatic effects of PPIs[458-459] on *H. pylori*. The effects of PPIs on *C. difficile*-associated diseases have, however, been contradictory; some studies have found that PPI use is a risk factor for *C. difficile* infection[460], whereas others argue that a causal relationship has not been established[461]. On the other hand, studies on the effects of PPIs on commensal microbiota are scarce. Since prolonged use of PPIs increases the gastric pH, microbes such as β-hemolytic streptococci – which cause pneumonia – may flourish in the stomach, thereby triggering increased susceptibility to infection and disease[462].

1.4.5 Health status & living conditions

Hospitalization of Scottish elderly people has been reported to result in a decrease of fecal *Bacteroides – Prevotella* group and *Ruminococcus albus* prevalence, while increasing the *Enterococcus faecalis* prevalence, as compared to healthy elderly people. Moreover, when the hospitalization was combined with antibiotic treatment, reductions in *Bifidobacterium* spp., *Desulfovibrio* spp., and *Clostridium butyricum* were also observed[456]. Long-term residential care of Irish elderly people has been associated with higher proportion of bacteria belonging to the phylum Bacteroidetes and the genera *Parabacteroides, Eubacterium*, *Anaerotruncus*, *Lactonifactor*, and *Coprobacillus*, whereas community-dwelling elderly people had higher proportions of bacteria belonging to the phylum Firmicu-

tes and genera *Coprococcus* and *Roseburia*. In addition, the microbiota of elderly people in long-term care was significantly less diverse. However, the results also correlated with the diet; if the elderly people were grouped according to their diet, the clustering was similar to that based on the residence location. After one month of residential care, the diet was converged to "long-term diet", but it took a year for the microbiota to clearly cluster within the long-term residential type. Moreover, this phenomenon was clearly seen in those elderly people who attended the hospital as out-patients or stayed briefly in the rehabilitation hospital care. The microbiota, as well as the diet, of these two latter groups was an intermediate between the long-term residential care and community-dwelling groups of elderly people. Collectively this indicates that the composition of the fecal microbiota is determined rather by the composition and diversity of the diet than by the location of residence[345].

Stress may also alter the fecal microbiota. It has been shown that anger stress increases the numbers of *Bacteroides thetaiotaomicron*, whereas emotional stress (with no anger) decreases the numbers of fecal lactobacilli and bifidobacteria[463-465].

1.5 The role of microbiota in certain diseases and disorders

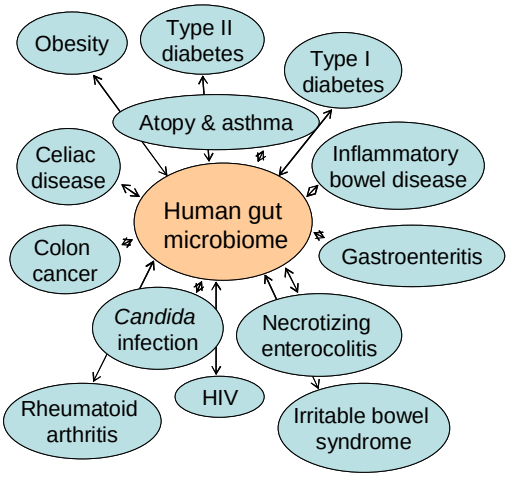

Figure 9. Diseases and disorders associated with human gut microbiome aberrations (adapted from[466]).

Recently gut microbiota has been implicated in numerous diseases and/or disorders (Figure 9). However, in most of the cases only correlations or associations have been shown, rather than causality. The diseases caused by a certain pathogen fulfilling Koch's postulates are not discussed in detail in this section. Only those diseases and/or disorders that are proposed to be caused by disturbances in the "normal gut bacterial ecology" are discussed in more detail.

1.5.1 Oral diseases

The major infectious diseases of the oral tissue are all polymicrobial[20]. **Dental plaque** is a multispecies biofilm displaying community characteristics such as tertiary structures with aqueous or exopolysaccharide-filled channels. In addition, microbes of dental plaque have physiological co-dependencies and quorum sensing[467-469]. The oral primary colonizing bacteria – *Streptococcus* and *Actinomyces*- form the basis for the dental plaque. These provide a base biofilm for the subsequent attachment and accumulation of other bacteria – e.g. *Veillonella* and other *Actinomyces* – that form close metabolic relationships with streptococci. Thereafter *Porphyromona*s and other streptococci are able to attach, as the local microenvironment start to favor anaerobic bacteria as well. Anaerobic conditions also enable the incorporation of *Fusobacterium*, which interacts with a wide range of bacteria involved in plaque formation and has, therefore, been suggested to be an important bridging component in oral microbial communities. As *Porphyromonas* and *Fusobacterium* are incorporated into the biofilm, they most likely also facilitate the growth of *Treponema*, which is part of the so-called "red-group" of periodontal pathogens. Furthermore, when the host immunity is compromised, yeasts (e.g. *Candida albicans*) may also be included in the sub-gingival plaque community[20,467]. Dental plaque that is not removed forms a basis for polymicrobial oral diseases and if systemically distributed, even for cardiovascular, musculo-skeletal and nervous system diseases[20,470-471].

Dental caries activity causes lesions and cavities on tooth surfaces, which lead to decay and even loss of tooth structure, resulting in infection and pain. The prevalence of dental caries is high; 92% of US adults aged 20 to 64 have had dental caries in their permanent teeth (http://www.nidcr.nih.gov/DataStatistics). Dental caries lesions are known to contain a variety of streptococcal species, lactobacilli, actinomyces, prevotellae, and occasionally *Candida* yeasts[20,30,472-473]. Moreover, the abundance of the genus *Prevotella* is higher in caries microbiota and the *Prevotella* species differ from those of healthy individuals[25]. In the oral cavity, indigenous lactobacilli and bifidobacteria do not have the same positive image as in the gut: *Bifidobacterium*-like organisms have been detected in advanced carious lesions[472] and oral lactobacilli are implicated as contributory bacteria in demineralization of the teeth once carious lesions are established[474]. There are three major hypotheses for the etiology of dental caries: 1) the specific plaque hypothesis, according to which only a few specific species (e.g. *S. mutans*) are causative agents[20,475-476]; 2) the non-specific plaque hypothesis, in which it is proposed that caries is an outcome of the overall activity of the plaque microbiota[476-477]; 3) the ecological plaque hypothesis, in which it is proposed that a change in key environmental factors will trigger a shift in the balance of the resident plaque microbiota, and this might predispose a site to disease[25, 31,476,478]. Although there are three different hypotheses for the microbial involvement in dental caries, the medical consensus is that acids from microbial fermentation of carbohydrates obtained from the diet result in an imbalance of the demineralization and remineralization processes on the enamel[479].

Gingivitis, an inflammation of the gum tissue, is a non-destructive periodontal disease. It is associated with the accumulation of plaque around the gingival margin[476]. Estimates of the general prevalence of adult gingivitis vary from approximately 50 to 100% for dentate subjects[480]. The subgingival microbiota of gingivitis differs from that of both healthy tissue and advanced periodontitis[481]. Bacteria involved in the etiology of gingivitis include species of *Streptococcus*, *Fusobacterium, Actinomyces, Veillonella*, and

Treponema, and possibly *Prevotella*, *Capnocytophaga*, and *Eikenella*[481-482]. If gingivitis is not treated, it may progress to periodontitis[482].

Periodontal disease (periodontitis) is a polymicrobial infection that destroys the attachment fibers and supporting bone that hold the teeth, being the most common cause of tooth loss worldwide[469]. It is caused by several microbial species, of which *Porphyromonas gingivalis*, *Tannerella forsythensis*, and *Treponema denticola* are considered to be the "red-complex" pathogens showing strong association with the disease, particularly in tobacco smokers and elderly people. In addition, e.g. *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Prevotella intermedia, Treponema socranskii,* and potentially also oral lactobacilli and *Selenomonas* spp. have been associated with periodontitis[20,32,216,259,469,483-487].

1.5.2 Irritable bowel syndrome (IBS)

IBS is an intestinal disorder that involves continuous or recurrent intestinal pain or discomfort that is relieved during defecation. In addition, IBS symptoms include altered stool frequency, form or passage, and passage of mucus and bloating[488]. Diagnostic criteria of IBS are based on symptoms and according to the Rome III criteria they are: "Recurrent abdominal pain or discomfort at least 3 days per month in the last 3 months associated with 2 or more of the following: 1) Improvement with defecation; 2) Onset associated with a change in frequency of stool; 3) Onset associated with a change in form (appearance) of stool. Criteria should be fulfilled for the last 3 months with symptom onset at least 6 months prior to diagnosis". Moreover, people having IBS are further divided into subtypes depending on the predominant stool pattern; 1) constipation type IBS; 2) diarrhea type IBS; 3) mixed type IBS; and 4) unclassified IBS (does not meet the criteria for any of the subtypes)[489]. The etiology of IBS is unknown, but genetic, biological, and psychosocial factors may be involved[489-490]. Moreover, some overlapping similarities have been found between IBS and inflammatory bowel disease (IBD), namely: similar genetic factors predisposing to IBD have also been associated with IBS; IBS and IBD both have impaired gut barrier function; and stress-induced flares and activation of mast cells typical for IBD are also associated with some of the IBS types[491].

The existence of abnormal colonic fermentation in IBS[492], and alleviation of IBS symptoms by eradication of small intestinal bacterial overgrowth by antibiotic therapy[493], suggest that intestinal microbiota may have a role in IBS. In addition, there are many studies in which differences in the fecal microbiota and/or temporal stability have been reported[65,355,377,494-514]. Reduction in fecal bifidobacteria of IBS subjects has been a common finding with both culture-based and molecular techniques[355,494,498,500,510,514]. In addition, in most of the studies the numbers / relative abundance of bacteria of the class Gammaproteobacteria[497,508,513,515] and numbers of *Veillonella* spp.[498,512-513] have also been shown to be higher in IBS subjects than inhealthy controls. Temporal instability and lower biodiversity in fecal microbiota of IBS subjects have also been reported[65,497,505,507-508]. However, the results regarding other bacterial groups have been partly contradictory; e.g. numbers / relative abundance of *Bacteroides* spp. / Bacteroidetes have been reported to be lower in IBS subjects as compared to healthy controls in several studies[505,513-514], whereas in one study higher numbers and diversity of Bacteroidetes were observed in IBS subjects than in healthy controls[510].

1.5.3 Inflammatory bowel disease (IBD)

Inflammatory bowel diseases (IBD), e.g. Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory diseases, for which chronic and relapsing inflammation of the gastrointestinal tract is typical. In UC, the inflammation is localized in the colon mucosa and occurs in the proximity of the epithelium. Therefore, colonocytes are implicated in the pathogenesis of UC. In CD, the inflammation may occur in both ileum and colon. The symptoms of IBD vary from bloody diarrhea and weight loss to ulceration, perforation, and complete obstruction of the GI tract. The chronic nature of UC also increases the risk of colon cancer. The incidence of UC is 1.2 to 20.3 cases per 100,000 persons per year, and its prevalence is 7.6 to 246 cases per 100,000 per year, as compared to the incidence and prevalence of CD; 0.03 to 15.6 and 3.6 to 214 per 100,000 per year, respectively. The highest incidence and prevalence of IBD are seen in the North European and North American populations. Western diet in addition to westernized lifestyle and environment, e.g. medication use, stress, smoking, and high socioeconomic status have been linked with high prevalence of IBD. Since germ-free mice do not develop colitis, the gastrointestinal microbiota is thought to be one of the most important environmental factors associated with IBD[516]. Most of the genes currently thought to be involved in the pathogenesis of IBD [nucleotide-binding oligomerization domain 2 (NOD2), autophagy-related protein 16-1 (ATG16L1), and immunity-related GTPase family M protein (IRGM)], are related to interactions between the immune system and the microbiota[517-519].

There are several common findings with regard to the gut microbiota of both UC and CD patients. The abundance of mucosa-associated bacteria of IBD patients has been shown to be higher than that of healthy adults, and it has been hypothesized that the capability of rejecting fecal bacteria in healthy mucosa has been profoundly disturbed in patients with IBD[496,520]. On the other hand, the mucosa-associated microbiota of IBD patients has also been shown to have reduced bacterial diversity and lower incidence of Firmicutes and Bacteroidetes bacteria, namely e.g. *F. prausnitzii, Clostridium nexile*, and *B. thetaiotaomicron*, whereas the incidence of several Alpha-, Beta-, and Gammaproteobacteria has increased[263,521]. Contrary to these results, Walker *et al.*[522] found an increase in Bacteroidetes in IBD patients. In addition, fecal microbiota of both UC and CD patients has been found to have an unusual bacterial composition as compared to healthy controls, e.g. the numbers of Enterobacteria are increased and the numbers of lactobacilli are decreased in IBD patients. Moreover, Gammaproteobacteria and Epsilonproteobacteria, and in particular (adherent-invasive) *E. coli*, *Campylobacter concisus*, and enterohepatic *Helicobacter*, have all been associated with the pathogenesis of IBD[59, 523-528]. Besides the shared microbial characteristics, both diseases have their own distinct microbial features. Mucosa-associated microbiota of CD patients has been reported to have a reduced incidence of *F. prausnitzii* and increased incidence of *Mycobacterium avium* subsp. *paratuberculosis*, *Ruminococcus torques*, and *E. coli,* particularly the adherent-invasive *E. coli* (AIEC) pathovar[529-532]. Fecal microbiota of CD patients has been shown to be temporally unstable and to have reduced bacterial diversity, especially of the *C. leptum* group[59,523, 528,533-534] and lower numbers of bifidobacteria[59,524] as compared to the healthy controls. Fecal microbiota of UC patients has been reported to have reduced numbers of *E. rectale* group bacteria[523].

The role of SRB – through production of H_2S – in the mucosal inflammation has been hypothesized for several decades. Butyrate is the preferred fuel of the colonocytes and it also plays a major role in the regulation of cell proliferation and differentiation. Hydrogen sulfide impedes butyrate oxidation in colonocytes, and therefore it has been implicated in the etiology of IBD[384,535-536]. Due to the contradictory results obtained hitherto[527,537-539], clear association with SRB and IBD has not been established. However, H_2S may still be an environmental factor contributing to the etiology of IBD, since it has been found that the H_2S production rates were higher in SRB isolated from patients with ulcerative colitis as compared to those isolated from healthy controls[539].

1.5.4 Allergy and asthma

An altered (both in numbers and composition) bifidobacterial population in infants has been associated with allergies, asthma, and atopic dermatitis[540-544]. The number of bacteria within Bacteroidaceae was significantly higher in the allergic group (at the ages of 1 and 2 months) than in the healthy controls[545-546]. In addition, the relative abundance of the genera *Propionibacterium* and *Klebsiella* (at the age of 2 months) was higher, whereas the relative abundance of the genera *Acinetobacter* and *Clostridium* as well as the numbers of lactobacilli were lower than in the healthy controls[543,546].

Children, who have developed asthma before the age of three years, have been shown to harbor higher numbers and prevalence of *B. fragilis* at the age of three months than the healthy controls[547]. In addition, colonization at three weeks with either *Bacteroides* spp. or *E. rectale* group members was associated with increased asthma risk[548].

Atopic children have been reported to have more bacteria from the Clostridial cluster I and fewer bifidobacteria in their fecal samples than non-atopic children. In addition, children with atopic eczema have been reported to have a less diverse total microbiota, less diverse genus *Bacteroides* (at the age of 1 month), lower counts of bacteroides (at the age of 12 months), and less diverse phylum Proteobacteria (at the age of 12 months) as compared to healthy children, whereas the prevalence and counts of *E. coli* have been reported to be higher in atopic children[289,321,540,549-552]. However, in another study less diverse fecal microbiota was associated with increased risk of allergic sensitization, allergic rhinitis, but not with asthma or atopic dermatitis[553].

1.5.5 Obesity, metabolic syndrome and diabetes

Obesity is a complex health issue with serious consequences such as type 2 diabetes (T2D), cardiovascular diseases etc. Obesity, metabolic syndrome, and T2D are all characterized by low-grade inflammation. There are several factors that contribute to the development of obesity; namely genetic, environmental, behavioral, and psychosocial factors[554]. After the findings of Turnbaugh *et al.*[555] and Ley *et al.*[82] that the relative proportion of Bacteroidetes decreased and the relative proportion of Firmicutes increased in obese mice[555-556] and men[82] as compared to their lean counterparts, obesity-related gut microbiota studies have attracted considerable attention. There have been several related studies in which the findings have been similar[83,557-560] to those of Ley *et al.*[82,556] and Turnbaugh *et al*.[555]. Moreover, there have also been studies in which the findings have

been contradictory or there have not been any statistically significant differences between Firmicutes and Bacteroidetes in obese/over-weight and normal weight people[345,378,381,561-564], and therefore the relationship between Firmicutes and Bacteroidetes vs. obesity still remains controversial. Maukonen *et al.*[237] showed that the numbers of Gram-positive bacteria are greatly underestimated when the "golden-standard" DNA-isolation kit is applied to fecal samples. Most of the obesity-related studies that have obtained similar results, i.e. that the relative proportion of Bacteroidetes decreases and the relative proportion of Firmicutes increases in obese humans[82] have used QIAmp DNA Stool Mini Kit[558-560,562], 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[563-565] or the samples have been studied with fluorescent *in situ* hybridization[378,381,561,564]. Therefore, the differences in the DNA-extraction step may, at least partly, explain the contradictory results. Obese people have been reported to have less diverse microbiota, phylum-level changes in the microbiota, and altered representation of bacterial genes and metabolic pathways[83]. In addition, an altered (both in numbers and composition) bifidobacterial population in infants[566], as well as the use of antibiotics[567], have been associated with obesity. Since bifidobacteria are susceptible to many antibiotics, it is also possible that the use of antibiotics causes the reduction of bifidobacteria, which is then detected in the obese children. Furthermore, it has not been shown whether these changes are due to the diet leading to obesity or to obesity itself, since there are no studies in which the microbiota of people gaining weight has been followed. Moreover, it has been shown in mice studies that absence of intestinal microbiota does not protect from diet-induced obesity[568] and that high-fat diet determines the composition of the mice microbiota independently of obesity[569]. The impact of the diet should, therefore, also be considered. In addition, it has been shown in twin studies that the amount of food consumed is the major contributor to obesity independently of genetic predisposition[570]. However, the gut microbiota has the potential to influence host physiology and behavior in ways that may have an impact on weight gain, fat deposition, and metabolic health, since SCFAs are known to interact with host receptors with the potential to influence gut hormones, inflammation, gut motility, and satiety[571].

Metabolic syndrome may be defined as a group of health problems, the common fundamental pathogenic component of which is resistance to insulin. Metabolic abnormalities include glucose intolerance (T2D, impaired glucose tolerance, or impaired fasting glycaemia), insulin resistance, central obesity, dyslipidemia, and hypertension. When all these symptoms are grouped together, they are associated with increased risk of cardiovascular disease. The prevalence of metabolic syndrome is highly age-dependent, i.e. metabolic syndrome is more common in the middle age to elderly age groups than in younger adults. However, since the prevalence of obesity in adolescents and young adults is increasing, we may also expect that the prevalence of metabolic syndrome in young adults will rise in the future[572]. There have been several microbiological findings related to people having metabolic syndrome; they have been reported to have higher proportion of bacteria belonging to *E. rectale – B. coccoides* group as detected with FISH[573], and lower diversity of *Bacteroides* spp. as detected with DGGE[312].

T2D is a metabolic disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency. Adults with T2D have been reported to have a lower proportion of Firmicutes, especially those belonging to the Class Clostridia,

whereas the proportions of the classes Bacilli and Betaproteobacteria were highly enriched in the diabetic subjects. The ratio of Bacteroidetes to Firmicutes and the proportion of the genus *Prevotella* correlated positively, whereas the proportion of the genus *Roseburia* negatively correlated with the values of the plasma glucose as detected with pyrosequencing. Moreover, the ratio of *Bacteroides – Prevotella* group to *E. rectale – B. coccoides* group also correlated positively with the values of the plasma glucose, and the counts of the *Lactobacillus* group were higher in the diabetic subjects when qPCR analysis was applied[285]. In addition, people having T2D have been reported to have lower numbers of bifidobacteria[574] and *F. prausnitzii*[575]. Interestingly, low *F. prausnitzii* numbers have also been associated with other inflammatory diseases, e.g. IBD[576].

Type 1 diabetes (T1D) is an autoimmune disease resulting from T-cell mediated destruction of insulin-producing β-cells. Much of the T1D etiology is accounted for by genetic predisposition. However, since the incidence of T1D has increased rapidly in Western countries during the past few decades, environmental factors may also play an important role[577]. Besides genetic and environmental factors, an aberrant intestinal microbiota, a "leaky" intestinal mucosal barrier, and altered intestinal immune responsiveness have also been proposed to have a role in the onset of T1D[578]. Children with T1D have been reported to have less diverse and stable microbiota compared to healthy children as detected with pyrosequencing. In addition, the proportion of a single species, *Bacteroides ovatus*, was greatly increased, whereas the proportion of a certain Firmicutes strain (no further phylogenetic position was given) was decreased in the children with T1D[579]. However, in this study[579] no Actinobacteria were found in the samples of Finnish children who were part of the DIPP-study, even though representatives from the classes Betaproteobacteria, Erysipelotrichi, and Fusobacteria were found. Maukonen *et al.*[312] have analyzed samples from 76 Finnish children who were also part of the same DIPP study and had the same HLA genotype (altogether 212 samples at similar ages as in Giongo *et al*.[579]), and bifidobacteria were found in nearly all of the samples (96% of the children;[312]). The samples of Giongo *et al*.[579] were also subjected to metagenomic analysis[303], in which it was found that the control children had higher proportions of butyrate-producing and mucin-degrading bacteria, whereas the children with T1D had higher proportions of those bacteria that produce other SCFAs than butyrate. Interestingly, bifidobacteria were found to be among the 11 most abundant genera on the basis of metagenomic analysis, although they were not detected with pyrosequencing[303]. These contradictory results indicate that the DNA-extraction method used was capable of extraction of high G+C Actinobacteria, but that for some reason the pyrosequencing step excluded them.

1.5.6 Others

Besides the aforementioned diseases and/or disorders, there are also many others in which gut microbiota has been implicated to have a role. However, these will only be briefly discussed. The role of gut microbiota in some psychiatric disorders has also been speculated. Autism is a complex disorder with unknown etiology. Besides genetic factors, environmental factors that may contribute to the etiology of autistic disorders include pre- or postnatal exposure to certain environmental chemicals and drugs, stress, maternal infection, and dietary factors[580]. There are several studies in which *Clostridium bolteae* and bacteria from Clostridial clusters I and XI have been shown to be more abundant in

autistic children than in healthy control children[581-583]. In addition, the bacterial diversity of autistic children has been found to be higher with a pyrosequencing study; the prevalence of bacteria belonging the phyla Bacteroidetes and Proteobacteria and the genera *Acetanaerobacterium*, *Alkaliflexus*, *Bacteroides, Desulfovibrio*, and *Parabacteroides* were higher in autistic children, whereas the prevalence of bacteria belonging to the phyla Firmicutes and Actinobacteria and 14 genera e.g. *Anaerofilum*, *Clostridium*, *Ruminococcus*, *Streptococcus*, and *Weissella* were lower in the autistic children[286]. Children with autism have also been found to have lower numbers of *Bifidobacterium* spp., *Enterococcus* spp., *Klebsiella oxytoca* and higher numbers of *Lactobacillus* spp. and *Bacillus* spp.in feces using standard culture-based techniques[584]. The lower levels of bifidobacteria as well as *A. muciniphila* have also been noted with a qPCR-based study[585], as have the higher levels of *Sutterella* spp.[586].

Colorectal cancer (CRC), also known as large bowel cancer, is one of the most common forms of cancer. Cancer manifests as the uncontrolled proliferation of host cells. The etiology of cancer is not completely clear, and as in other diseases/disorders in which the role of gut microbiota has been studied, both genetic and environmental factors have been shown to have an effect on the onset of the cancer, although diet is considered to be the most significant risk factor by many researchers[587]. There are several studies in which the correlation between the gut microbiota and CRC has been studied and in most of them some kind of microbiota dysbiosis has been detected. However, in different studies the observed dysbiosis has been partly different; with qPCR-based studies the number of bacteria belonging to the *Bacteroides – Prevotella* group has been higher in the CRC patients[588], whereas in a pyrosequencing-based study it was found that *B. fragilis* type of OTUs were enriched in the cancer patients and *B. vulgatus* and *B. uniformis* type OTUs were enriched in the healthy controls[587]. Furthermore, OTUs belonging to the genera *Enterococcus*, *Escherichia*/*Shigella*, *Klebsiella*, *Streptococcus*, and *Peptostreptococcus* were enriched in the cancer patients, whereas OTUs belonging to the genera *Roseburia* and other butyrate-producers within Lachnospiraceae were less abundant. Moreover, qPCR results validated the lower counts of butyrate-producing bacteria in the cancer patients[587].

In addition to the characterization of the fecal microbiota of the CRC patients, tumor microbiota has also been studied. *Fusobacterium* spp. sequences have been found to be enriched in carcinomas, confirmed by quantitative PCR and 16S rRNA gene sequence analysis of 95 carcinoma/normal DNA-pairs, whereas the phyla Bacteroidetes and Firmicutes were depleted in tumors[589]. Moreover, genera over-represented in tumors included members of the Coriobacteridae, *Roseburia*, *Fusobacterium*, and *Faecalibacterium*, whereas potentially pathogenic enterobacteria were under-represented in tumor tissue[590].

Celiac disease is a chronic inflammatory disorder of the small intestine, with permanent intolerance to cereal gluten proteins. Chronic inflammation in the small intestine results in villous atrophy and flattening of the mucosa. Treatment with a gluten-free diet usually leads to remission of clinical symptoms and normalization of mucosal histology. Celiac disease may manifest at any age in those with genetic susceptibility, but in many cases the symptoms already start in childhood[591]. In addition, cesarean delivery has been found to correlate positively with the onset of celiac disease[592]. Since environmental factors also have a role in the manifestation of celiac disease, the role of gut microbiota in its etiology has been studied. It has been found that children with celiac disease have higher bacterial diversity[593-594], lower numbers of bifidobacteria and higher numbers of *C. leptum*

group bacteria and *Bacteroides* spp. in both feces and duodenal biopsies, and lower numbers of *C. histolyticum* group and *C. lituseburense* group bacteria and *F. prausnitzii* in feces than healthy children. Moreover, the counts of *E. coli* and *Staphylococcus* spp. were higher in the untreated children, but decreased to the same level as those of the healthy children after treatment with a non-gluten diet[595-598]. Similar results have also been obtained with a culture-based study; children with celiac disease were found to have lower numbers of bifidobacteria and lactobacilli and higher numbers of *Bacteroides* spp., *Clostridium* spp., *Salmonella* spp., *Shighella* spp., and *Klebsiella* spp.[599-600]. However, the levels of *Staphylococcus* spp. varied between the culture-based studies; in the first study the levels of *Staphylococcus* spp. were lower in the children with celiac disease[599], whereas in the second, healthy children had lower levels of *Staphylococcus* spp.[600]. The effect of gluten-free diet on healthy adults has also been studied to assess the effects caused by the gluten-free diet. It was observed that the numbers of *Bifidobacterium* spp., *C. lituseburense* group, *Lactobacillus* group, and *F. prausnitzii* decreased, whereas the numbers of Enterobacteriaceae and *E. coli* increased. Simultaneously the production of TNF-α, interferon-γ, IL-10, and IL-8 by peripheral blood mononuclear cells was reduced. Thus, the gluten-free diet resulted in reduction of beneficial bacteria in the GI-tract[601].

2. Hypotheses, rationale and specific aims of the study

This study was based on four hypotheses. The first hypothesis was that the developed molecular tools enable rapid and cost-effective characterization of the human predominant fecal microbiota. A large fraction (>80%) of the GI-tract microbiota has not yet been cultured, which necessitates the use of molecular techniques in the characterization of GI-tract microbiota. However, due to the immense numbers of bacteria in fecal samples, the optimization and validation of the methods applied to fecal samples is of utmost importance.

The second hypothesis was that the fecal microbiota of people having IBS differs from that of healthy adults, and that the difference is distinguishable with molecular microbiological techniques. At the time the study was conducted, the results relating to the fecal microbiota of people having IBS were partly contradictory and only a few studies had been published using molecular microbiological techniques. Most of the members of the Clostridial clusters XIV and IV are difficult to culture, especially from fecal samples when specific enumeration and characterization is needed. Therefore it was expected that more insight into the possible role of the most predominant Clostridial clusters of the human fecal microbiota in IBS would be gained.

The third hypothesis was that the fecal microbiota of elderly people differs from that of younger adults and that the elderly fecal microbiota may be modified with a GOS-yoghurt intervention. The gut microbiota evolves with age, and the differences in the gut microbiota of elderly people and younger adults have been shown previously. However, the results have been partly contradictory and dependent on the geographical location of the subjects. Therefore, the aim was to assess the differences within the Finnish population. Moreover, although the effects of prebiotics on the fecal microbiota have been studied, GOS has received less attention. Since bifidobacteria have been shown to be present in lower numbers in elderly people, the ingestion of bifidogenic GOS could in theory increase the abundance of bifidobacteria.

The fourth hypothesis was that there might be confounding factors responsible for the contradictory results obtained in many recent studies – especially differences in storage conditions and DNA-extraction protocols of fecal samples. There are a few studies in which bifidobacteria were found to be a minor part of the infant microbiota, whereas there are numerous studies in which bifidobacteria have been found to be a predominant part of the infant microbiota with both culture-based and culture-independent methods. Moreover, especially in the obesity related studies the results have been contradictory. The hypothesis was that some of the used DNA-extraction methods were not capable of ex-

tracting Gram-positive bacteria efficiently, especially those belonging to the phylum Actinobacteria. Moreover, since the gastrointestinal tract is an open system, the salivary and upper gastrointestinal tract microbiota may also influence the results obtained with DNA-based methods. Therefore the similarity of the salivary and fecal microbiota was studied.

The primary aim of this study was to characterize human predominant fecal microbiota with a special focus on Clostridial clusters XIV (Lachnospiraceae, *Eubacterium rectale – Blautia coccoides* group) and IV (Ruminococcaceae, *Clostridium leptum* group).

The specific aims were:

- To develop molecular methods for characterization of the human predominant fecal microbiota
- To assess the specificity, practicality, and usability of the developed methods for human fecal samples in healthy adults, elderly people, and people having IBS.
- To assess possible confounding factors in the analysis of human fecal samples.

3. Materials and methods

3.1 Materials

3.1.1 Bacterial pure cultures

The bacterial pure cultures used in this study for optimization and validation of molecular methods are presented in Table 1. The bacterial strains were grown as recommended by the culture collection in question. However, the methanogens (*Methanobrevibacter smithii* and *Methanosphaera stadtmanae*) were not grown at VTT, but the extracted DNA was purchased directly from DSMZ.

Table 1. Bacterial pure cultures used in the studies.

Article	Species	Strain	Clostridial Cluster [a]
V	*Alistipes finegoldii*	VTT E-093113	
V	*Anaerofilum agile*	DSM 4272	IV
V	*Anaerofilum pentosovorans*	DSM 7168	IV
II, V	*Anaerostipes caccae*	VTT E-052773	XIV
V	*Anaerotruncus colihominis*	VTT E-062942	IV
II, V	*Atopobium parvulum*	VTT E-052774	
V	*Bacteroides caccae*	VTT E-062952	
V	*Bacteroides dorei*	DSM 17855	
V	*Bacteroides egghertii*	VTT E-093118	
V	*Bacteroides finegoldii*	VTT E-093115	
II, V	*Bacteroides fragilis*	VTT E-022248	
V	*Bacteroides intestinalis*	VTT E-093114	
V	*Bacteroides ovatus*	VTT E-062944	
V	*Bacteroides plebeius*	DSM 17135	
V	*Bacteroides thetaiotaomicron*	VTT E-001738	
V	*Bacteroides vulgatus*	VTT E-001734	
V	*Bifidobacterium adolescentis*	VTT E-981074	
V	*Bifidobacterium angulatum*	VTT E-001481	
V	*Bifidobacterium animalis* subsp. *animalis*	VTT E-96663	
V	*Bifidobacterium animalis* subsp. *lactis*	VTT E-97847	
IV	*Bifidobacterium animalis* subsp. *lactis*	Bb-12	

V	*Bifidobacterium breve*	VTT E-981075	
V	*Bifidobacterium catenulatum*	VTT E-11764	
V	*Bifidobacterium longum* subsp. *infantis*	VTT E-97796	
II, V	*Bifidobacterium longum* subsp. *longum*	VTT E-96664	
II, V	*Blautia coccoides*	VTT E-052778	XIV
II, V	*Clostridium acetobutylicum*	VTT E-93498	I
V	*Clostridium beijerinckii*	VTT E-93498	I
II, V	*Clostridium bolteae*	VTT E-052776	XIV
II, V	*Clostridium butyricum*	VTT E-97426	I
II, V	*Clostridium clostridioforme*	VTT E-052777	XIV
V	*Clostridium hathawayi*	VTT E-062951	XIV
II, V	*Clostridium histolyticum*	VTT E-052779	II
II, V	*Clostridium indolis*	VTT E-042445	XIV
II, V	*Clostridium leptum*	VTT E-021850	IV
II, V	*Clostridium lituseburense*	VTT E-021853	XI
II, V	*Clostridium perfringens*	VTT E-98861	I
V	*Clostridium sporosphaeroides*	VTT E-062947	IV
II, V	*Clostridium symbiosum*	VTT E-981051	XIV
II, V	*Collinsella aerofaciens*	VTT E-052787	
V	*Desulfobacter curvatus*	VTT E-001657	
V	*Desulfobacterium autotrophicum*	VTT E-001658	
V	*Desulfosarcina variabilis*	VTT E-001656	
V	*Desulfotomaculum nigrificans*	VTT E-001654	
II, V	*Desulfovibrio desulfuricans* subsp. *desulfuricans*	VTT E-95573	
V	*Desulfovibrio piger*	DSM 749	
V	*Desulfovibrio vulgaris* subsp. *vulgaris*	VTT E-95573	
II, V	*Dorea longicatena*	VTT E-052788	XIV
II, V	*Eggerthella lenta*	VTT E-001735	
II, V	*Enterococcus faecalis*	VTT E-93203	
II, V	*Enterococcus faecium*	VTT E-93204	
II, V	*Escherichia coli*	VTT E-94564	
II, V	*Eubacterium eligens*	VTT E-052844	XIV
II, V	*Eubacterium hallii*	VTT E-052783	XIV
II, V	*Eubacterium ramulus*	VTT E-052782	XIV
V	*Eubacterium siraeum*	VTT E-062949	IV
V	*Faecalibacterium prausnitzii*	DSM 17677	IV
II, V	*Fusobacterium necrophorum*	VTT E-001739	
II, V	*Fusobacterium nucleatum* subsp. *nucleatum*	VTT E-052770	
II, V	*Klebsiella terrigena*	VTT E-96696	
II, V	*Lachnospira multipara*	VTT E-052784	XIV
IV	*Lactobacillus acidophilus*	LaCH-5	
V	*Lactobacillus brevis*	VTT E-91458	
V	*Lactobacillus casei*	VTT E-85225	
V	*Lactobacillus paracasei*	VTT E-93490	
V	*Lactobacillus plantarum*	VTT E-79098	
V	*Lactobacillus rhamnosus*	VTT E-97800	
II, V	*Lactobacillus salivarius*	VTT E-97853	

II, V	*Megasphaera elsdenii*	VTT E-84221	IX
V	*Methanobrevibacter smithii*	DSM 861	
V	*Methanosphaera stadtmanae*	DSM 3091	
V	*Parabacteroides distasonis*	VTT E-062943	
V	*Parabacteroides merdae*	VTT E-062953	
V	*Parascardovia denticolens*	VTT E-991434	
II, V	*Pectinatus cerevisiiphilis*	VTT E-79103	IX
II, V	*Pediococcus acidilactici*	VTT E-93493	
V	*Prevotella intermedia*	DSM 20706	
II, V	*Prevotella melaninogenica*	VTT E-052771	
II, V	*Roseburia intestinalis*	VTT E-052785	XIV
V	*Ruminococcus obeum*	VTT E-052772	XIV
II, V	*Ruminococcus productus*	VTT E-052786	XIV
V	*Scardovia inopinatum*	VTT E-991435	
II, V	*Selenomonas lacticifex*	VTT E-90407	IX
V	*Subdoligranulum variabile*	VTT E-062950	IV
II, V	*Veillonella parvula*	VTT E-001737	

[a] Clostridial phylogenetic cluster number[60].

3.1.2 Bacterial isolates

Parts of the fecal samples from which bifidobacterial and lactobacilli isolates were obtained (Article IV) were transferred to Cary-Blair transport medium[602] and parts of the salivary samples to VMGA III medium[603] and analyzed by culture within 1–2 days. The following culture media and incubation conditions were used (incubations at 37°C): supplemented Brucella blood agar (Tammer Tutkan maljat) for anaerobes (anaerobic incubation for 7 d), sheep blood agar for aerobes (Tammer Tutkan maljat) (aerobic, 4 d), and Beerens[604] for bifidobacteria (anaerobic, 4 d). For the detection of *B. animalis* subsp. *lactis* Bb-12, the samples were also acid pre-treated[605]. Rogosa agar was used for the detection of lactobacilli (anaerobic, 3 d). For the detection of *L. acidophilus* LaCH-5 the Rogosa plates were incubated microaerophilically. Beerens and Rogosa agars were also used with tetracycline supplementation (8 µg/ml), since *B. animalis* subsp. *lactis* Bb-12 is intermediately resistant to tetracycline. Isolates representing all different potential *Lactobacillus* and *Bifidobacterium* colony morphologies or, in the case of uniform colony morphology, random isolates were collected from Beerens- and Rogosa-based media (if possible 5 isolates from each medium) for comparison of the species distribution in saliva and feces.

3.1.3 Human sample collection and handling

The clinical materials collected for different studies are presented in Table 2.

The main recruiting criterion for “healthy people” (Articles I–V) was a 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 two months prior to the study. The studies of healthy people were approved by the ethical committee of VTT Technical Research Centre of Finland. All subjects gave their written informed consent to participation in the study.

The IBS group (Article I) comprised 16 subjects, who fulfilled the Rome II criteria for IBS[488]. All patients had undergone clinical investigation and endoscopy or barium enema of the gastrointestinal tract 0–1 years prior to the study. Exclusion criteria were pregnancy, breast feeding, organic intestinal diseases or other severe systematic diseases, previous major or complex abdominal surgery, severe endometriosis, antimicrobial medication during the previous two months, and dementia or otherwise inadequate co-operation. Patients with lactose intolerance were included if they reported following a low-lactose or lactose-free diet. The IBS study was approved by the human ethics committee at the joint authority for the hospital district of Helsinki and Uusimaa (HUS).

The elderly group (Article III) consisted of 41 elderly subjects with self-reported constipation. Additional inclusion criteria were; age between 60 and 80 years and a successful completion of the Mini-Mental State questionnaire[606]. The exclusion criteria were daily use of laxatives, other gastrointestinal disorders (celiac disease, IBD, gastrointestinal tumors, diverticulitis, or IBS), thyroid dysfunction, use of strong psychopharmaceuticals or opioids, and use of antimicrobials during the preceding month. The Human Ethics Committee of the Joint Authority for the Hospital District of Helsinki and Uusimaa (HUS, Finland) approved the study protocol. All subjects gave their written informed consent to participation in the study.

The subjects defecated into a plastic container, which was then 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 and processed in an anaerobic workstation (Don Whitley Scientific Ltd., Shipley, UK) within 0–4 h of defecation (Articles I, II, IV, V). The samples were maintained at -70^{o}C until analyzed. In the study of Article III, the subjects defecated into a plastic container and placed the samples immediately after defecation into their own freezer (-20^{o}C). Within one week, the samples were transferred into a -70^{o}C freezer to wait for further analysis.

3.2 Methods

3.2.1 Nucleic acid extraction and biotinylation (Articles I–V)

DNA was extracted from 200 mg of fecal material using FastDNA Spin Kit for Soil (QBIOgene, Carlsbad, CA, USA) according to the manufacturer's instructions with one modification; the bacterial cells were disrupted with a Fast Prep instrument at 6.0 m/s for 60 s three times.

For DNA-extraction experiments (article V) 6 different protocols (FastPrep lysis: 1) 60s 4.5 m/s; 2) 60s 6.5 m/s; 3) 60s + 30s 6.5 m/s; 4) 60s + 60s s 6.5 m/s; 5) 60s + 60s + 30s 6.5 m/s; 6) 60s + 60s + 60s 6.5 m/s) with FastDNA SpinKit for Soil (QBIOgene) and two different protocols: (1) protocol for Gram-negative bacteria; 2) protocol for Gram-positive bacteria) with QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) were evaluated. The isolated DNA was stored at -20^{o}C until examined.

RNA was extracted from reference strains and feces as previously described by Satokari *et al.*[228] and further purified by using the clean-up protocol of RNeasy mini kit (Qiagen). RNA concentration and purity were determined spectrophotometrically (A260/A280; BioPhotometer 6131, Eppendorf, Hamburg, Germany) and the integrity of RNA was eval-

uated with agarose gel electrophoresis in which 23S and 16S rRNA were observed. Purified RNA used for quantitative analysis (TRAC; see below) was biotinylated by using Photoprobe® Biotin (Vector Laboratories, CA, USA). Biotinylation was performed by exposing the reaction to long wave UV-light (365 nm) for 30 min and the subsequent purification of the RNA from free biotin was performed according to the manufacturer's instructions as previously described[228].

3.2.2 PCR and reverse transcriptase (RT) –PCR (Articles I–V)

Partial 16S rRNA genes of the bacterial group of interest were PCR-amplified with the primers specified in Table 3 and with conditions specified in the Article in question. RT-PCR was performed with the Qiagen® OneStep RT-PCR Kit according to the manufacturer's instructions with or without a Q-solution. Q-solution modifies the melting behavior of nucleic acids thus facilitating reverse transcription and amplification of templates with a high GC content or a high degree of secondary structure. Primers used for the RT-PCR were the same as were used for the PCR (Table 3).

3.2.3 Denaturing gradient gel electrophoresis (DGGE; Articles I–V)

PCR products were separated in polyacrylamide gels with a denaturing gradient of 38–60% (predominant bacteria and Erec-group), 45–55% (bifidobacteria and *B. animalis* subsp. *lactis* Bb12), or 30–60% (*Lactobacillus*-group, Clept-group and *Bacteroides* spp.) (where 100% is 7 M urea and 40% (vol/vol) deionized formamide) as described previously[497]. *L. acidophilus* LaCH-5 was added as a probiotic control lane to each *Lactobacillus*-group specific DGGE (in Article IV).

Similarity of the PCR-DGGE-profiles of the samples obtained from a single subject at different sampling points was compared in order to evaluate the temporal stability of selected fecal bacterial populations. Comparison of the profiles was performed by calculating a similarity percentage using BioNumerics software version 4.01 (Articles I–IV) or version 5.1 (Article V) (Applied Maths BVBA, Sint-Martens-Latem, Belgium). 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.

Table 2. Clinical study material.

Article	Study	Subjects	Sampling	Age and sex distribution
I	IBS vs. healthy adults	Subjects with IBS and healthy adults	Fecal samples; baseline and 6 months	control group; 4 males and 12 female subjects, 26–63 years of age (mean 45 years) IBS-group; 5 male and 11 female subjects, 24–64 years of age (mean 45 years)
II	Erec-DGGE method development	Healthy adults	Fecal samples; baseline and 6 months (subjects 5–12). For a longer-term stability study; baseline, 3, 6, and 24 months (subjects 1–4)	3 male and 9 female subjects, 34–63 years of age
III	Elderly people	Elderly people suffering from constipation and healthy adults	Fecal samples (elderly study group); baseline and 21 days GOS-yoghurt intervention Fecal samples (elderly placebo group); baseline and 21 days yoghurt intervention Fecal samples (healthy adults); baseline	41 elderly subjects with self-reported constipation (10 male and 31 female subjects, 60 to 79 years of age; mean 68 years) Healthy adult volunteers from Articles I and II; The two subjects whose age 60 years or more were excluded from the previous study groups.
IV	fecal vs. salivary bacterial community	Healthy adults	Fecal and salivary samples; baseline, 1week probiotic intervention, 2 weeks probiotic intervention	1 male and 9 female subjects, 34–57 years of age
V	DNA-extraction optimization + method development	Healthy adults	Fecal samples; baseline (two adults) Fecal samples; baseline, 3 months and 6 months (10 adults)	two healthy adults for DNA-extraction optimization (female, 44 years old and male, 51 years old) and 10 healthy adults (3 males and 7 females, 34–62 years of age) for *Clostridium leptum* group as well as *Bacteroide*s spp. specific DGGE validation

3.2.4 Cloning (Articles II & V)

PCR amplicons were purified using a Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions. DNA purity and yield were estimated by electrophoresis in 1% (w/v) agarose gels. Purified amplification products were ligated into pGEM-T vector system II and transformed into *Escherichia coli* JM109 high efficiency competent cells (Promega, Madison, WI, USA) according to the manufacturer's instructions. 130–146 colonies of ampicillin-resistant transformants were randomly picked from each sample. PCR with pGEM-T-specific primers T7 and SP6 (Table 3) was performed from the lyzed cells to check the size of the inserts. The PCR-products of the plasmids containing inserts of the correct size were purified with the Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions and sequenced with the ABI PRISM BigDye terminator Cycle sequencing kit v.3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions using the primer T7. All the sequences were analyzed with ABI PRISM 3100 automated capillary DNA cycle sequencer (Applied Biosystems).

3.2.5 Sequence analysis (Articles II & V)

The sequences were checked and edited with the Chromas program (Technelysium Pty Ltd, Helensvale, Australia). ClustalW (http://align.genome.jp; Article II or http://www.ebi.ac.uk/Tools/clustalw2/index.html?; Article V) was used for the alignment of the sequences and for checking of the sequence similarities.

All unequal sequences were thereafter identified through the GenBank database (www.ncbi.nlm.nih.gov) using the "BLAST" (Basic Local Alignment Search Tool) algorithm[607] or using the “Classifier” tool of the Ribosomal Database Project (RDP) II[134]. ClustalW and DNAMAN 4.1 (Lynnon BioSoft) were used for the creation of operational taxonomic units (OTU) in Article II. An OTU, as employed here, consisted of all sequences with less than 2% divergence from 440 aligned homologous nucleotides[61]. Phylogenetic analysis of OTU subgroups was performed with DNAMAN 4.1. One representative of each OTU was deposited in the GenBank database and the sequences are available under the accession numbers DQ307759-DQ307802. “Library compare” of RDPII [608] was used for the classification of sequences derived from DNA extraction optimization (Article V) 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-JN20712. Phylogenetic analyses were performed with the Kodon software (Applied Maths, Sint-Martens-Latem, Belgium).

3.2.6 Fingerprinting of bacterial isolates (Article IV)

Randomly amplified polymorphic DNA (RAPD) was used for fingerprinting of bifidobacteria and lactobacilli (Article IV). 587 isolates from Beerens were analyzed using primer OPA-2 (Table 3) and 574 isolates from Rogosa were analyzed using primer OPA-3 (Table 3) as previously described[605]. The RAPD fingerprints of fecal and salivary isolates were compared to those of the *B. animalis* subsp. *lactis* Bb12 and *L. acidophilus* LaCH-5

strains by visual inspection. All types that appeared to be different from the ingested probiotic strains were selected for further characterization.

3.2.7 Real time PCR (qPCR; Article V)

Partial 16S rRNA genes of the bacterial group of interest were qPCR-amplified with the primers specified in Table 3 and with conditions specified in Article V. All the qPCRs were performed in duplicate (from duplicate DNA-extractions; i.e. four replicates in all). Standard curves were created as described in Article V.

3.2.8 Transcript analysis with the aid of affinity capture (TRAC; Articles I and II)

Multiplexed quantification of clostridial 16S rRNA was performed with the TRAC technique using specific 16S rRNA targeted probes for different groups of clostridia and a universal bacterial probe (Table 3)[228]. The probes were labeled with 6-FAM (6-carboxyfluorescein) at the 5'-end and HPLC-purified. RNA (maximum amount from five to ten ng) was denatured at 70^{o}C for 2 min and hybridized with all the oligonucleotide probes at 50^{o}C for 1h. After hybridization the biotin-nucleic acid-probe complexes were captured on magnetic streptavidin-coated microparticles and washed. The hybridized probes were eluted and their identity and quantity were determined by capillary electrophoresis with an ABI PRISM 310 Genetic Analyzer. The signal intensities of the recorded probes corresponded to the amount of target nucleic acid in the mixture, while the size indicated the target. Results were expressed as percent of total bacterial 16S rRNA that was detected by the Bact338-IIA probe and were given as means ± standard deviations from triplicate measurements.

3.3 Statistical analysis (Articles I–V)

qPCR and culture data were transformed into logarithmic scale in order 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 variances" or "two-sample assuming unequal variances", depending on the dataset) was used for statistical analysis of the results obtained from a single DGGE-analysis. Multivariate analysis of variance (MANOVA) and principal component analysis (PCA), included in the BioNumerics software (Applied Maths BVBA), were used for statistical analysis of the composite data sets. In addition, discriminant analysis including similarity between baseline and first intervention (Article III) and difference in band numbers was performed with SPSS 14.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

Table 3. Primers (PCR-DGGE or qPCR) and probes (TRAC) used in this study.

Target group	Short name	Probe / primer	Use	Sequence (5' → 3')	Reference or Source	Article
predominant bacteria[a]	Univ	U968-f +GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACG GGGGGAACGCGAAGAACCTTA	267	I–V
predominant bacteria[a]	Univ	U1401-r	PCR-DGGE	CGGTGTGTACAAGACCC	267	I–V
predominant bacteria[b]		358F + GC	PCR-DGGE	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACG GGGGGCCTACGGGAGGCAGCAG	266	
predominant bacteria[b]		358F	qPCR	CCT ACG GGA GGC AGC AG	266	V
predominant bacteria[b]		534R	PCR-DGGE qPCR	ATTACCGCGGCTGCTGG	266	V
predominant bacteria		Bact338-IIA	TRAC	GCTGCCTCCCGTAGGAGTIIA	609 & 228	I
Eubacterium rectale – *Blautia coccoides* group[c]	Erec-group	Ccoc-f	PCR-DGGE	AAATGACGGTACCTGACTAA	250	II–V
Eubacterium rectale – *Blautia coccoides* group[c]	Erec-group	Ccoc-f + GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACG GGGGGAAATGACGGTACCTGACTAA	242	II
Eubacterium rectale – *Blautia coccoides* group[c]	Erec-group	Ccoc-r	PCR-DGGE	CTTTGAGTTTCATTCTTGCGAA	250	II
Eubacterium rectale – *Blautia coccoides* group[c]	Erec-group	Ccoc-r + GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACG GGGGGCTTTGAGTTTCATTCTTGCGAA	242	II–V
Eubacterium rectale – *Blautia coccoides* group[c]	Erec-group	g-Ccoc-F	qPCR	AAATGACGGTACCTGACTAA	251	V
Eubacterium rectale – *Blautia coccoides* group[c]	Erec-group	g-Ccoc-R	qPCR	CTTTGAGTTTCATTCTTGCGAA	251	V
Eubacterium rectale – *Blautia coccoides* group[d]	Erec-group	Erec482-5A	TRAC	GCTTCTTAGTCARGTACCGAAAAA	15 & 228 251	I
Clostridium leptum group[e]	Clept-group	Clept-933 f	PCR-DGGE	GCACAAGCAGTGGAGT	251	V

Clostridium leptum group[e]	Clept-group	Clept-1240-r		GTT TTR TCA ACG GCA GTC	62	V
Clostridium leptum group[e]	Clept-group	Clept-1240-r +GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACG GGGGGGTTTTRTCAACGGCAGTC	237	V
Clostridium leptum group[e]	Clept-group	Clept-f	qPCR	GCACAAGCAGTCGAGT	251	V
Clostridium leptum group[e]	Clept-group	Clept-R3	qPCR	CTTCCTCCGTTTTGTCAA	251	V
Clostridium leptum group[e]	Clept-group	Clept1240	TRAC	GTTTTRTCAACGGCAGTC	62	I
Clostridium lituseburense group[f]	Clit-group	Clit135	TRAC	GTTATCCGTGTGTACAGGG	15	I
Clostridium histolyticum group[g]	Chis-group	Chis150	TRAC	TTATGCGGTATTAATCTYCCTTT	15	I
genus *Bacteroides*	*Bacteroides* spp.	Bact596f	PCR-DGGE		58	V
		Bacto1080r		GCA CTT AAG CCG ACA CCT	73	V
genus *Bacteroides*	*Bacteroides* spp.	Bacto1080r + GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACG GGGGGGCACTTAAGCCGACACCT	237	V
genus *Bacteroides*	*Bacteroides* spp.	g-Bfra-F	qPCR	ATAGCCTTTCGAAAGRAAGAT	251	V
genus *Bacteroides*	*Bacteroides* spp.	g-Bfra-R	qPCR	CCAGTATCAACTGCAATTTTA	251	V
Bifidobacteria	Bif	Bif164-f	PCR-DGGE seq,	GGGTGGTAATGCCGGATG	256	III–V
Bifidobacteria	Bif	Bif662-GC-r	PCR-DGGE seq,	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACG GGGGGCCACCGTTACACCGGGAA	256	III–V
B. animalis subsp. lactis Bb12	Bb12	Bif164mod-f	PCR-DGGE	GGGTGGTAATACCGGATG	610	IV
Bifidobacteria	Bif	Bifid-f	qPCR	CTCCTGGAAACGGGTGG	250	V
Bifidobacteria	Bif	Bifid-r	qPCR	GGTGTTCTTCCCGATATCTACA	250	V

Lactobacillus-group[h]	Lac	Lac1	PCR-DGGE	AGCAGTAGGGAATCTTCCA	58	III–V
Lactobacillus-group[h]	Lac	Lac2GC	PCR-DGGE	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCG CCCCCGCCCCATTYCACCGCTACACATG	58	III–V
Atopobium-group[f]	Ato	Atopo-f	qPCR	GGGTTGAGAGACCGACC	251	V
Atopobium-group[f]	Ato	Atopo-r	qPCR	CGGRGCTTCTTCTGCAGG	251	V
sequencing (clones)		T7	seq.	TAATACGACTCACTATAGG	Promega	II, V
sequencing (clones)		SP6	seq.	GATTTAGGTGACACTATAG	Promega	II, V
sequencing (16S rRNA)		BSF8/20	seq.	AGAGTTTGATCCTGGCTCAG	611	IV
sequencing (16S rRNA)		BSR1541/20	seq.	AAGGAGGTGATCCAGCCGCA	611	IV
bifidobacteria fingerprinting		OPA-2	RAPD	TGCCGAGCTG	605	IV
lactobacilli fingerprinting		OPA-3	RAPD	AGTCAGCCAC	605	IV

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

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

[c] Clostridial phylogenetic cluster XIVa[60]

[d] Clostridial phylogenetic clusters XIVa & XIVb[60]

[e] Clostridial phylogenetic cluster IV[60]

[f] Clostridial phylogenetic cluster XI[60]

[g] Clostridial phylogenetic clusters I & II[60]

[h] *Lactobacillus*-group includes genera such as *Lactobacillus, Leuconostoc, Pediococcus*, and *Weissella*

[f] *Atopobium*-group includes genera such as *Atopobium*, *Eggerthella*, and *Collinsella*

4. Results and discussion

4.1 Development of bacterial group-specific PCR-DGGE- and qPCR-methods (Articles I, II, and V)

A large fraction (>80%) of the GI-tract microbiota has not yet been cultured[55], which necessitates the use of molecular techniques in the characterization of GI-tract microbiota. The predominant intestinal clostridia mainly belong to clusters XIVa (*E. rectale – B. coccoides* group) and IV (*C. leptum* group)[15,55,61-64], whereas most of the medically important clostridia belong to cluster I[612]. Many clostridia are cultivable, but their culture-based quantification is impractical due to unavailability of good sub-group selective media (with the exception of some clinically important species). Hence, culture-independent techniques have great practical relevance in the study of clostridia. Clostridia contain saccharolytic and proteolytic species and they are able to metabolize a wide variety of substrates. In the colon they have an important role in the fermentation and putrefaction of food-derived substances, resulting in various metabolites such as fatty acids and gases. Short-chain fatty acids formed by microbial fermentation have an important role in colonic health; butyrate plays a role in the metabolism and normal development of colonic epithelial cells. Butyrate has also been implicated in protection against cancer and ulcerative colitis[51]. 80% of the butyrate-producing isolates of human fecal origin have been shown to belong to the clostridial phylogenetic cluster XIVa[396].

Bacteroides spp. were also added as a target group in this study, since Bacteroidetes have been shown to be one of the predominant groups in the human fecal microbiota[15,62-64,73-74], although the focus was on Clostridial clusters XIV and IV.

4.1.1 rRNA-based vs. rDNA-based detection

rRNA-based methods were developed in this study to enable the detection of the metabolically active fraction of the fecal microbiota. Since DNA remains relatively intact in non-viable cells, methods targeting DNA enumerate both viable and dead cells[188-189]. RNA is more labile than DNA and more susceptible to degradation caused by deleterious conditions/treatments[190]. Due to the fact that environmental conditions influence the cellular rRNA content, the amount of rRNA in cells is considered to correlate with the growth rate[191].

rRNA-based DGGE methods were developed for the predominant bacteria (Article I) and *E. rectale – B. coccoides* group (Article II). Overall, the rRNA-based DGGE-profiles contained less amplicons than the DNA-based DGGE-profiles; in predominant bacterial DGGE-profiles, the rRNA-based profiles contained on average 15 less amplicons ($p<0.05$), whereas in the *E. rectale – B. coccoides* group DGGE-profiles the difference was on average only one amplicon. A clear difference in almost all of the samples between the DNA-based and rRNA-based predominant bacterial DGGE-profiles was the lack of high GC-amplicons in the rRNA-based DGGE-profiles. The differences were also seen in all parts of the denaturing gradient. The rRNA- and DNA-based profiles had such different appearances that they were easily distinguishable. Moreover, the rRNA-based profiles of different time points were more similar to each other than the DNA-based and rRNA-based profiles of a given time point. The clear difference between the DNA- and RNA-derived predominant fecal bacterial populations has also been reported previously[239].

The difference between the DNA- and rRNA-based DGGE-profiles of *E. rectale – B. coccoides* group was less clear. The number of amplicons on both rRNA- and DNA-based DGGEs was similar, and in most cases rRNA- and DNA-based DGGE-profiles of a given sample were more similar than the intra-individual temporal stability as detected with either DNA- or rRNA-based DGGE.

The predominant bacterial DGGE results obtained in Article I indicated that the use of rRNA-based DGGE would give more insight into the active part of the fecal microbiota than DNA-based methods. However, when the results of *E. rectale – B. coccoides* group DGGE in Article II indicated that the rRNA- and DNA-based profiles greatly resembled each other, we did not continue with the rRNA-based DGGE-method development. In seemed that since the rRNA molecule remains stable for some time after cell death, the rRNA content of a bacterium may not correctly reflect its physiological status. Thus the laborious and expensive approach was discontinued.

4.1.2 *Eubacterium rectale – Blautia coccoides* group (Clostridial cluster XIV; Lachnospiraceae) PCR-DGGE

The *E. rectale – B. coccoides* group (Erec) is one of the major bacterial groups in human feces[15,61,201], and according to one study within this thesis (Article I[65]), the most predominant Clostridial cluster in fecal samples of Finnish people. At the time Article II was published, only group-specific quantitative methods were available[15,59,228,251-252,582]. Recently there have also been other studies in which the Erec-group has been characterized with microarrays or high-throughput sequencing[210-211,565]. Since it was extensively demonstrated that the Erec-group bacteria are abundant in the human fecal microbiota, a simple PCR-DGGE method, which does not necessitate the construction of a clone library from each sample, was developed to assess the diversity of this group. The method was optimized with a wide number of different bacteria (Table 1, Article II) and further validated with a clone library. All the 205 clones belonged to the Erec-group. The 108 clones of

subject 4 fell into 21 OTUs (Figure 1, Article II) and the 97 clones of the subject 6 into 22 OTUs (Figure 2, Article II), when an OTU was defined as a phylogenetic group consisting of members having over 98% sequence similarity to each other from 440 aligned homologous nucleotides. Moreover, all the amplicons seen in the DGGE-profiles were identified through the clone library and each DGGE band was comprised of either one OTU or a few OTUs that were phylogenetically closely related. Therefore the number of amplicons seen in the DGGE-profiles reflected the phylogenetic diversity of the samples. In addition, the method proved to be semi-quantitative, since the intensity of an amplicon correlated with the clones retrieved at that position (Figures 1 and 2, Article II). The majority of the sequences retrieved from the 205 clones of the two subjects were different. Similar findings have also been observed in other studies[55]. However, when the cloned samples were run in the same DGGE-gel, several amplicons migrated similarly (Figure 1, Article II). The two samples for cloning were chosen on the basis of the abundance of the Erec-group bacteria – one sample contained a high percentage of Erec-group bacteria and another a low percentage. Our results showed that the number of bacteria belonging to the Erec-group was not related to the diversity of that same bacterial group. One sample (subject 4, baseline sample: Figure 1, Article II) containing 71% of Erec-group bacteria had 15 DGGE bands representing 21 OTUs, whereas another sample containing 27% of Erec-group bacteria (subject 6, baseline sample; Figure 2, Article II) had 16 DGGE bands representing 22 OTUs.

After method validation, diversity and temporal stability of Erec-group bacteria from fecal samples of healthy adults were evaluated. Although considerable intra-individual diversity as well as uniqueness of the predominant fecal bacterial population with both DNA- (24–51 amplicons per each sample) and RNA-based (12–25 amplicons per each sample) PCR-DGGE analyses were observed, the same intra-individual diversity was not observed in Erec-group DGGE-profiles (12–18 amplicons/DNA-sample; 10–17 amplicons/RNA-sample), even though this group comprised a majority of the microbiota in some individuals according to the hybridization results. However, since it is possible to detect only those bacteria that comprise over 1% of the total population with DGGE[266], we were able to detect the majority of the Erec-group bacteria, as could be seen from the clone library results. The inter-individual uniqueness of Erec-profiles was less clear than that of the predominant bacterial profiles. Regardless of this, samples of a single subject clustered together in most cases. In addition, the clone libraries of the two subjects indicated that even though the DGGE-profiles resembled each other and contained several similar OTUs, only less than 20% of their sequences were identical, which is in agreement with the findings of Tap *et al.*[81] and Turnbaugh *et al.*[284].

The Erec-group was found to be temporally as stable or unstable as the predominant bacteria, which was as expected since the Erec-group is the most abundant bacterial group detected with the predominant bacterial DGGE. In the two-year stability study the DGGE-profiles of two subjects were unstable and two subjects had minor differences with both predominant bacterial DGGE and Erec-DGGE. In the shorter follow-up study (6 months), nearly all of the profiles that were unstable with either RNA-based or DNA-based Erec-DGGE, were also un-

stable with predominant bacterial DGGE. However, the hybridization-detected proportional stability was not found to correlate with the DGGE-based temporal stability. This finding further addresses the difference between proportion and diversity of a given bacterial group discussed above.

4.1.3 *Clostridium leptum* group (Clostridial cluster IV; Ruminococcaceae) PCR-DGGE

The *C. leptum* group (Clept) has been shown to be the second most abundant Clostridial cluster of Finnish fecal microbiota[65]. Therefore, a DGGE-method was developed to cost-effectively study the diversity and temporal stability of this bacterial group. The primer pair Clept-933 f – Clept-1240-r + GC gave positive PCR results for all the target bacteria belonging to the Clept-group. No false positive results were obtained. In most cases only one strong or one strong and one additional weak band was/were seen in the DGGE-profiles of the reference strains. All amplicons of the reference strains of the Clept-group migrated differently and could thus be distinguished (data not shown). Two samples were cloned after the Clept-group PCR to validate the specificity of the method. All the 204 sequenced Clept-group PCR clones belonged to the *C. leptum* Clostridial cluster IV. Notably, over half of the sequences clustered with *F. prausnitzii* when a phylogenetic tree was constructed using the neighbor-joining algorithm (data not shown). Clept-group specific DGGE proved to be semi-quantitative; the stronger the band, the more clones were found migrating into that position. Moreover, single clones were not seen in the profile, which supported the theory that only bacteria comprising over 1% of the targeted population may be detected with DGGE[266]. The majority of the sequences retrieved from the 204 clones of the two subjects were different. This is in agreement with previous studies[55]. However, when the cloned samples were run in the same DGGE-gel, several amplicons migrated similarly. The DGGE-profiles of the cloned samples and the sequence information obtained from the cloning of Clept-group are presented in Figures S1 and S2 of Article V.

After the method validation, the diversity and temporal stability of Clept-group bacteria from fecal samples of healthy adults were evaluated. Considerable intra-individual diversity as well as uniqueness was found. The Clept-group diversity was on average 20.9 ± 3.4 amplicons (range 16–28 amplicons per sample). Intra-individual similarity of the follow-up samples (three and six months) was higher than inter-individual similarity, i.e. in 8/10 subjects all the samples from a given subject clustered together (each individual created his/her own cluster; data not shown). The intra-individual similarity of samples taken three months apart was 87.5 ± 9.2%, whereas the intra-individual similarity of samples taken six months apart was on average 81.7 ± 11.7%.

4.1.4 *Bacteroides* spp. PCR-DGGE

The proportion of Bacteroidetes has varied greatly between different studies, depending on the used probe and thus the target to be quantified. In this study, *Bacteroides* spp. were chosen instead of Bacteroidetes, because *Bacteroides* spp. (formerly *Bacteroides fragilis* group) have been found in the lower intestine, whereas if we had targeted Bacteroidetes we could potentially also have detected the DNA of the oral *Prevotella* spp. and *Porphyromonas* spp. Presently (August, 2012) there are 47 described type species within the genus *Prevotella* (http://www.bacterio.cict.fr/index.html), only two of which have been isolated from feces, whereas 32 type strains have been isolated from the oral cavity, four from ruminants and one from a plant residue. However, there are five *Prevotella* spp. type strains which have been isolated from clinical infections, one from amniotic fluid, one from pleural fluid, and one from a breast abscess. The original habitats of these strains are not known, and therefore they may originate from any part of the oro-gastrointestinal tract. Moreover, in a recent study of elderly people in which the proportions of different genera within the phylum Bacteroidetes were given, the predominant genera were *Bacteroides*, *Alistipes*, and *Parabacteroides*, with no mention of *Prevotella*[278]. By targeting only *Bacteroides* spp. we did not target Rikenellaceae, which would have been targeted by choosing Bacteroidetes. The abundance of Rikenellaceae has not been shown to be very high in younger adults and thus their omission was preferred to the possibility of detecting oral bacterial DNA in feces.

The primer pair Bact596f – Bacto-1080r + GC gave positive PCR results for all the target bacteria belonging to the genus *Bacteroides*. No false positive results were obtained. In most cases only one strong or one strong and one additional weak band was/were seen in the DGGE-profiles of the reference strains. All amplicons of the reference strains of *Bacteroides* spp. migrated differently and could thus be distinguished (data not shown). Two samples were cloned after the *Bacteroides* spp. specific PCR to validate the specificity of the method. All the sequenced 240 *Bacteroides* spp. specific PCR clones belonged to *Bacteroides* spp. The DGGE-profiles of the cloned samples and the sequence information obtained from the cloning of *Bacteroides* spp. are presented in Figures S3 and S4 of Article V. As with previous DGGE-methods, *Bacteroides* spp. specific DGGE is also semi-quantitative; the stronger the band, the more clones were found migrating to that position. Moreover, single clones were not seen in the profile, which confirmed the theory that bacteria comprising over 1% of the targeted population may be detected with DGGE[266].

After method validation, the diversity and temporal stability of *Bacteroides* spp. were evaluated. Diversity of *Bacteroides* spp. was on average 9.2 ± 1.9 (range 5–12 amplicons per sample). Intra-individual similarity of the follow-up samples was higher than inter-individual similarity, i.e. in 8/10 subjects all the samples from a given subject clustered together (each individual created his/her own cluster; data not shown). The intra-individual similarity of *Bacteroides* spp. of samples taken three months apart was 85.4 ± 12.1%, whereas the intra-individual similarity of *Bacteroides* spp. of samples taken six months apart was on average 82.0 ± 16.2%.

4.1.5 qPCR methods

Since we showed in Article II that the proportion and diversity of a given bacterial group do not correlate with each other, we wanted to develop easier and faster quantification methods than TRAC. In TRAC, RNA-extraction, purification, and biotinylation are needed. In addition, the actual TRAC-procedure is very sensitive to manual errors and takes rather a long time to perform. We wanted to develop DNA-based rapid methods for the quantification of the most predominant bacterial groups, and therefore developed several qPCR-methods.

Primers (Table 3) were chosen based on *in silico* testing of published primer pairs. All the used qPCR-methods, targeting predominant bacteria, Clept-group, Erec-group, *Bacteroides* spp., bifidobacteria, and *Atopobium* group, were optimized with an extensive set of positive and negative controls (Table 2). The qPCR conditions were optimized in such a way that all target strains amplified after around 15 cycles and non-target strains after 35 cycles of the program. Reduction of one logarithm corresponded to approximately 4 cycles of qPCR. By using the aforementioned protocol we wanted to make sure that as little as possible unspecific binding could occur, since it is not feasible to validate methods with all possible gastrointestinal bacteria. Standard curves were created with the help of DNA extracted from a known number of culturable representatives; *Blautia coccoides* for predominant bacteria and Erec-group, *Anaerotruncus colihominis* for Clept-group, *B. fragilis* for *Bacteroides* spp., *B. longum* for bifidobacteria, and *A. parvulum* for *Atopobium* group.

4.2 Differences in the fecal microbiota between healthy adults and adults with IBS (Article I)

Some older studies have reported differences in fecal bacterial populations between healthy adults and those with IBS[494-495,497-498,613]. However, the results obtained in those studies were partly contradictory and with the exception of those of Mättö *et al.*[497] and Malinen *et al.*[498] were conducted with culture-based analysis. Therefore, the role of intestinal microbiota in IBS was still rather poorly known at the time of this study. Since IBS has often been linked with extensive gas production in the colon[492], and some bacterial groups are more prone to gas production than others, the possible role of the composition and/or activity of clostridia and related bacteria in intestinal (in)balance was studied.

4.2.1 Temporal stability of predominant bacterial populations and clostridial populations

Mättö *et al.*[497] showed using DNA-based analysis that people having IBS had more instability in their fecal predominant microbiota than healthy controls. We broadened the study by including rRNA-based analysis. DNA-based PCR-DGGE

and rRNA-based RT-PCR-DGGE analyses targeted to the predominant bacterial population showed considerable diversity as well as uniqueness of the microbiota in each individual in both control and IBS groups. Furthermore, the rRNA-based DGGE-profiles of IBS subjects were more unstable than those of the healthy controls. Neither DNA- nor rRNA-based predominant DGGE analysis revealed any IBS-specific amplicons. Both DNA- and rRNA-based predominant DGGE-profiles were heterogeneous, containing approximately 20–30 and 15–25 amplicons, respectively. The rRNA-based DGGE-profiles contained significantly less amplicons than the DNA-based DGGE-profiles ($p<0.05$; Fig. 1 in Article II). As can be seen in the Figure 1 of Article II, there were differences in all parts of the denaturing gradient. However, the amplicons with the highest GC-content, which migrate to the lower part of the DGGE gel, were generally missing in the rRNA-based DGGE-profiles. The clear difference between the DNA- and rRNA-derived predominant fecal bacterial populations was also reported by Tannock *et al.*[239]. The differences may be due to several reasons; the resolving power of RT-PCR is limited by the efficiency of RNA-to-cDNA conversion[614] and the DNA obtained from complex samples does not reflect the metabolic activity of the microbiota[615], whereas rRNA is regarded as an indicator of the total bacterial activity[268]. Therefore, the amplicons present only in the DNA-based profiles may reflect bacteria with low activity.

When an rRNA-based DGGE-profile was compared to a DNA-based DGGE-profile of the same sample, the temporal intra-individual similarity value was significantly lower than the intra-individual similarity value between DNA-based profiles or rRNA-based DGGE-profiles between different time points ($p<0.05$; Table 4). However, since each individual had a unique DNA- and rRNA-based DGGE-profile, the intra-individual profiles resembled each other more than the inter-individual RNA-based profiles (Fig. 1 in Article II). Moreover, the DNA-based DGGE-profiles of a certain person clustered together in nearly all of the cases, whereas with rRNA-based DGGE-profiles, samples from eight people having IBS and four healthy controls did not cluster together (data not shown).

Table 4. Similarity values of the DGGE-profiles obtained at two different time points (baseline and 6 months later). Similarity values were calculated with the BioNumerics 3.0 software. Clustering was performed with Pearson correlation and the UPGMA method. Amplicons with a total surface area of at least 1% were included in the similarity analysis.

	Qualitative DGGE analysis			
Subject	**RNA-based similarity (%; 1 vs. 2)**	**DNA-based similarity (%; 1 vs. 2)**	**Similarity (%; RNA1 vs. DNA1)**	**Similarity (%; RNA2 vs. DNA2)**
IBS subjects: diarrhea type				
Mean ± sd	72.3 ± 22.6	82.3 ± 17.9	76.2 ± 11.1	79.0 ± 11.4
Range	38.5–94.2	44.6–95.1	56.0–88.3	59.5–89.2
IBS subjects: constipation type				
Mean ± sd	72.4 ± 19.0	81.3 ± 7.1	68.3 ± 6.8	72.5 ± 10.8
Range	43.1–93.4	68.0–89.4	56.2–75.3	59.4–84.8
IBS subjects: mixed type				
Mean ± sd	86.1 ± 7.4	87.1 ± 1.3	75.6 ± 12.2	71.1 ± 8.8
Range	85–88.0	85.7–88.0	66.8–89.5	63.4–80.7
IBS subjects altogether				
Mean ± sd	75.1 ± 19.0	82.8 ± 12.3	73.1 ± 10.0	75.3 ± 10.7
Range	38.5–94.5	44.6–95.1	56.0–89.5	59.4–89.2
Healthy controls				
Mean ± sd	83.4 ± 10.8	84.8 ± 11.8	70.2 ± 16.7	72.1 ± 12.2
Range	68.2–94.2	58.8–96.7	31.1–88.7	42.4–86.4

The assessment of temporal stability of the predominant microbiota with DNA-based predominant bacterial DGGE did not reveal more instability in the IBS subjects than in the control group subjects, in contrast to the findings of Mättö *et al.*[497]. However, less similarity (i.e. more instability) was observed in the rRNA-based DGGE-profiles of the IBS subjects as compared to the healthy controls (Table 4). In addition, the similarity value range of rRNA-derived DGGE-profiles of people having IBS was much wider than that of the healthy controls (Table 4). The main difference between the study group of Mättö *et al.*[497] and the study group of this study was that people who had received antimicrobial therapy during the last two months prior to the two sampling time points were excluded in the latter, since antibiotic therapy causes disturbances in the intestinal microbiota[428,442,449,616] and may also affect the predominant bacterial groups[456]. Under normal circumstances predominant intestinal microbiota of an adult individual has been reported to be rather stable[56-59,242,617]. The effect of disease on the stability of intestinal microbiota is clearly seen e.g. in studies in which a marked instability of the predominant fecal microbiota has been observed in IBD patients[59,533,618]. In our study, people having temporal instability were observed in both IBS and control groups (with both DNA-

and RNA-based DGGE). In previous studies in which the long-term temporal stability of the predominant microbiota has been assessed from healthy people, the number of individuals has been limited (one to nine individuals)[56-59,242,617].

When people with IBS were divided into subgroups according to their symptoms, the greatest intra-individual variability in DGGE similarity values within the subject group was seen in the diarrhea type and the smallest in the mixed type (Table 4). The great intra-individual variation seen in the diarrhea type may be due to different phases of the syndrome; if a patient is experiencing a more symptomatic phase of IBS, the predominant bacterial population might be significantly different when compared to the symptom-free time, as has been reported for IBD patients[59]. However, we did not have data on the symptomatic activity of each IBS subject, and this conclusion therefore remains speculative. The smallest intra-individual change of the mixed type subject group in our study is at least partly explained by the small number of subjects (three); further studies are therefore needed with larger population groups.

No clear differences in intra-individual temporal stability (change in proportions between the two sampling points) of *C. leptum*, *C. lituseburense,* or *E. rectale – B. coccoides* groups were observed between the IBS and control subjects. However, *C. histolyticum* group was temporally more stable (the change in proportions of the *C. histolyticum* groups was smaller) in mixed type IBS subjects than in the control subjects ($p=0.05$). Altogether there was generally lower temporal than inter-individual variation in the studied clostridial groups, as was also reported by Matsuki *et al.*[251]. However, the instability of predominant bacteria was also seen in the change of the proportions of the studied clostridial groups in this study. People whose predominant bacterial population (as detected with rRNA-based DGGE) was unstable over the studied time period had a significant change in the clostridial proportions between the two sampling points (baseline and six months) when compared to the subjects whose predominant bacterial population was stable ($p<0.05$). This was as expected, since the studied clostridial groups represented the dominant fecal microbiota in these subjects. The same was also observed with DNA-based DGGE-profiles, but in the case of DNA-based profiles the difference was not statistically significant ($p=0.06$).

4.2.2 Proportion of clostridia

The studied clostridial groups (*E. rectale – B. coccoides*, *C. leptum, C. lituseburense,* and *C. histolyticum*) represented the dominant fecal microbiota of most of the studied subjects, which is in accordance with previous studies[61,63-64,277].

There were only small differences in the proportions of *C. leptum*, *C. lituseburense,* and *C. histolyticum* groups between people having IBS and healthy controls (Figure 8). It has been reported earlier that *C. lituseburense* and *C. histolyticum* groups comprise less than 1% of the total fecal microbiota of healthy adults[15,619-620]. However, in a recent study the proportions of *C. lituseburense* and *C. histolyticum* groups were found to be as high as 7.9% and 5.4%, respectively[601]. Our results for

C. lituseburense (mean 1.5–2.3%) and *C. histolyticum* group (mean 3–5% with corrected values) were thus well in accordance with the previously reported results. The proportions of the *C. leptum* group found in the healthy controls (mean 12% and 9%, for baseline and 6 month samples, respectively) and IBS subjects (mean 12% and 15%, for baseline and 6 month samples, respectively) were also in agreement with previous studies[62-64,70,276].

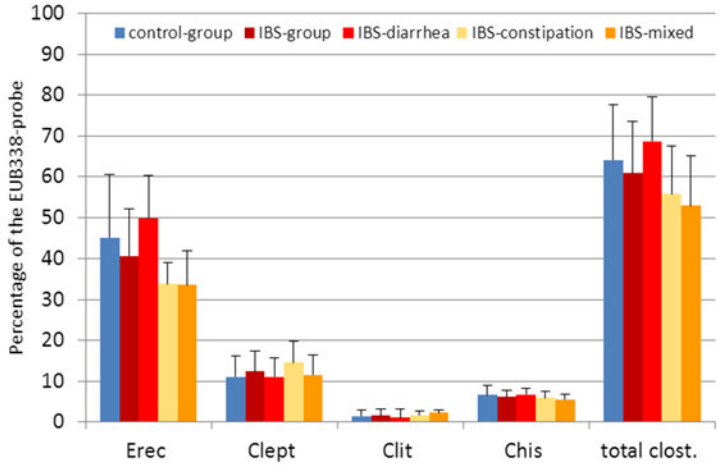

Figure 10. The proportions of different clostridial groups of IBS and control subject categories at baseline.

The Erec-group was the dominant subgroup of clostridia, accounting on average for 43% of the total bacteria in control subjects and 30% and 50% in irritable bowel syndrome (IBS) subjects affected by constipation and diarrhea, respectively. The proportion of the Erec-group was found to be significantly lower in the constipation type IBS subjects than in the control subjects ($p<0.05$; Figure 10). The average percentage observed with our healthy controls is somewhat higher than the previously reported 11–35% (range 5–45%) for healthy adults[15-16,62-64,66-67,69-70,276]. This highlights interindividual differences possibly due to diet and genetic background. In addition, the age distribution in both our subject groups was wider than in most of the other studies. As can be seen in Figures 11 and 12, the proportion of Erec-group was the lowest in healthy younger adults (20–29 years of age; 35%), whereas in the IBS group the lowest Erec-group proportion was found in the age group 50–59 (33%). It should be noted that smaller proportions of Erec-group compared to our results have generally been obtained with FISH, whereas the results of Rigottier-Gois *et al.*[67] obtained with dot blot hybridization were more similar to our study (range 12–55% vs. 18–71% in our study). The FISH analysis measures the proportion of the cells containing a sufficient number of ribosomes to be detected, whereas rRNA dot blot hybridization gives an index reflecting the ribosomal content of the cells and the general metabolic activity[67], as does the TRAC technique.

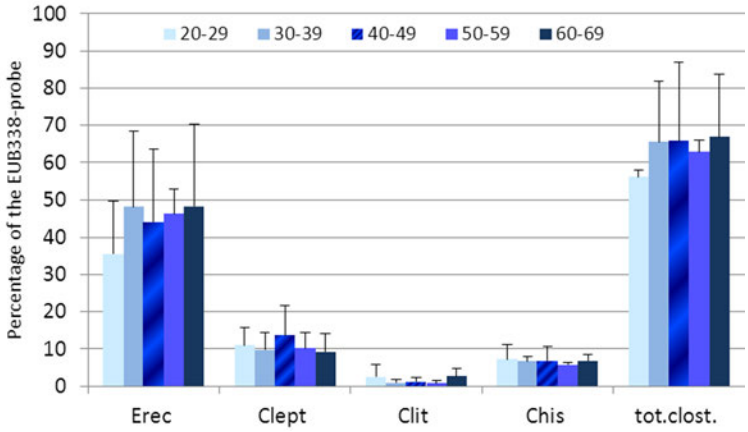

Figure 11. The proportion of clostridia (within the probe EUB338) in different age groups of healthy people, as detected with TRAC.

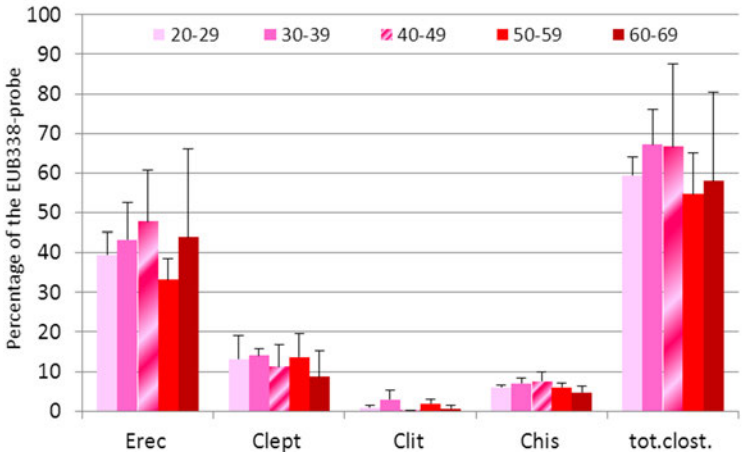

Figure 12. The proportion of clostridia (within the probe EUB338) in different age groups of people having IBS, as detected with TRAC.

No difference was observed in the present study in the proportions of Erec-group bacteria when IBS subjects altogether were compared to the control subjects. However, when different IBS symptom categories were compared to the control subjects the proportion of Erec-group bacteria was significantly lower in the constipation type IBS subjects than in the control subjects and also in the 6 months sampling time point of the mixed type IBS subjects ($p<0.05$) (Figure 10). Similar differences were observed by Malinen *et al.*[498] and Ponnusamy *et al.*[510]. By contrast, Swidsinski *et al.*[496] concluded that the numbers of cells belonging to the Erec-group were somewhat higher in the IBS group as compared to the control

group. The Erec-group (Clostridial cluster XIV) is too large of a target to detect subtle variations between microbiota of control and IBS subjects and therefore needs to be divided into smaller subgroups in the further studies.

4.3 Characterization of the fecal microbiota of elderly people during a GOS-yoghurt intervention (Article III)

It was already shown some decades ago that the gastrointestinal microbiota evolves with age[341]. The numbers of bifidobacteria have been reported to be lower in elderly subjects compared to younger adults in European studies[64,204,342,348-350]. Bifidobacteria are generally considered beneficial for health, and have widely been used both individually and in combination with lactobacilli in probiotic foods and products. The absence of bifidobacteria, or their low numbers in the elderly, may have metabolic and health consequences for the host, affecting immune system function and a multiplicity of other functions, e.g. synthesis of vitamins and protein, and supplementation in digestion and absorption[204, 347]. In addition, bifidobacteria are involved in colonization resistance in the bowel[204]. The number of bifidobacteria can be increased in the gut either by continuous supplementation of probiotic bifidobacteria or by adding prebiotics to food products. Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth, activity, or both of one or a limited number of bacterial species already resident in the colon[420]. Prebiotics include inulin, FOS, GOS, and lactulose[421]. GOS, derived from lactose in milk, is a collective term for a group of semi-synthetic non-digestible carbohydrates made using galactosidases as catalysts, and has received less attention than the other prebiotics, although they are considered to have bifidogenic effects in humans[239]. Since the number of bifidobacteria and the diversity of bifidobacterial populations have been reported to decrease with age, we wanted to study the effect of GOS-yoghurt on the bifidobacterial population of elderly people. Both the GOS- and placebo-yoghurts were fermented using a commercial starter culture containing *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, and *Bifidobacterium animalis* subsp. *lactis* strains.

4.3.1 Fecal microbiota differences between elderly people and younger adults

Temporal stability of the studied bacterial groups (predominant bacteria, Erec-group, lactobacilli, and bifidobacteria) did not differ between the elderly and younger adults, whereas an increase in the predominant bacterial diversity in elderly subjects was observed, as previously reported[211,278,343]. In addition, the inter-individual variation was greater in the elderly people as compared to the younger adults, as previously reported[14, 278,345]. The average number of predominant bacterial amplicons in the elderly people was significantly higher than that of

the younger adults (42 ± 5 vs. 32 ± 5 amplicons, respectively; p<0.05). In addition, the predominant bacterial DGGE-profiles of the elderly subjects were different according to visual inspection as compared to those of the younger adults. There were amplicons throughout the DGGE-profile (from top to bottom) derived from feces of the elderly subjects (Figure 2A, Article III), whereas in the DGGE-profiles of the younger adults (Figure 4A, Article III) there is usually a small empty gap between the upper part of the DGGE gel (low-medium GC-content; most of the adult fecal microbiota) and the lower part of the gel (high GC-content; bifidobacteria, Coriobacteriaceae, and sulfate-reducing bacteria).

Diversity of the Erec-group bacteria was significantly higher in the elderly subjects than in the younger adults (21 ± 4 vs. 15 ± 2 amplicons, respectively; p<0.05). The number of bacteria belonging to the Erec-group – as detected with FISH – has been shown to be higher in French, German, and Swedish elderly subjects as compared to younger adults and lower in Italian and Dutch elderly populations[64,348]. Since the Finnish diet is close to the Swedish diet, it could be expected that the numbers of bacteria belonging to the Erec-group would be higher in the elderly subjects than in the younger adults. We did not quantify the number of Erec-group bacteria, but we found significantly more amplicons from the elderly subjects than from the younger adults. In addition, since it was shown in Article II of this thesis that each amplicon corresponded to a phylogenetic cluster, the diversity of the fecal Erec-group of elderly subjects was higher than that of younger adults. Contradictory to these findings, as many operational taxonomic units (OTUs) of Erec-group bacteria have been found in Japanese elderly people as from the younger adults[621] and a reduction in *Ruminococcus* spp. and *Roseburia* spp. has been observed in Finnish elderly people[356]. However, some species of the genus *Ruminococcus* belong to the Erec-group (*R. gnavus, R. lactaris, R. obeum, R. torques*), whereas others belong phylogenetically to the Clept-group (*R. albus, R. bromii, R. cellulosi, R. callidus, R. flavefaciens*). The phylogenetic position of the *Ruminococcus* spp. was not specified in the article of Mäkivuokko *et al.*[356].

Diversity of bifidobacteria was similar in both elderly subjects and younger adults, which is not in agreement with the earlier culture-based studies[342,349]. However, in a more recent study there were no statistically significant differences in the abundance of bifidobacteria between elderly people and younger adults[64].

There was a trend that the diversity of *Lactobacillus*-group of the elderly subjects was somewhat higher (11 ± 4 amplicons) than that of the younger adults (8 ± 4; *P*=0.09). The abundance of lactobacilli has been shown to increase or stay stable in many studies, regardless of the nationality of the studied elderly people[211,255,347-348,352,355-356]. In addition, the fecal lactobacilli population of elderly subjects in this study was unstable, as has been reported for younger adults[58, 260].

4.3.2 The effect of GOS-yoghurt consumption on the fecal microbiota of elderly people

DGGE analysis targeted to the predominant bacterial population showed considerable intra-individual diversity as well as uniqueness of fecal microbiota before and after the intervention. The intra-individual fecal samples clustered together in all cases (one distinct cluster per subject), irrespective of GOS- or placebo-yoghurt administration. Predominant fecal microbiota was temporally rather stable after both placebo (mean similarity 81.6 ± 10.5%) and GOS-yoghurt consumption (mean similarity 80.9 ± 11.9%). In addition, the number of amplicons after GOS- and placebo-yoghurt consumption was similar to the number of amplicons at the baseline (Figure 2, Article III). This is in agreement with earlier studies, in which it has been found that GOS-containing biscuits had an effect on the RNA-derived predominant bacterial DGGE-profiles but not on the DNA-derived DGGE-profiles in New Zealand elderly people[239]. In addition, it was shown in one study of younger adults that the probiotics included in a test yoghurt did not have a great effect on the stability of the fecal predominant bacteria[186].

The intra-individual diversity and uniqueness of Erec-group DGGE in the fecal samples were similar to those of the predominant bacteria (Figure 2, Article III). Erec-group bacteria remained temporally mostly stable after both placebo (mean similarity 91.1 ± 6.1%) and GOS-yoghurt consumption (mean similarity 87.7 ± 7.9%). In addition, the number of amplicons after GOS- and placebo-yoghurt consumption was similar to the number of amplicons at the baseline.

The intra-individual diversity and uniqueness of bifidobacteria DGGE in the fecal samples were similar to those of the predominant bacteria and Erec-group bacteria (Figure 2; Article III). However, the intra-individual fecal samples clustered together in only 26/38 subjects (one distinct cluster per subject; data not shown). The subjects whose samples clustered together had an intra-individual temporal similarity value of greater than 85%. The fecal bifidobacterial population was temporally mostly rather stable after both GOS-yoghurt consumption (mean similarity 82.3 ± 21.7%) and after placebo-yoghurt consumption (mean similarity 82.0 ± 19.8%). The number of amplicons after GOS- and placebo-yoghurt consumption was similar to the number of amplicons at baseline. We used a PCR-DGGE which did not target the ingested *Bifidobacterium animalis* subsp. *lactis*[256,610], and therefore it did not affect the diversity or temporal stability of the fecal bifidobacterial profiles. In several other studies, increase in bifidobacterial abundance has been observed[288,370,423]. However, it was also shown in one study that even when GOS was administered for many weeks at high doses, some individuals still did not have a bifidogenic response[423]. This further addresses the question of responders and non-responders in probiotic and prebiotic research.

Lactobacillus-group targeted DGGE analysis showed intra-individual diversity as well as uniqueness of *Lactobacillus* microbiota before and after intervention (Figure 2; Article III). The intra-individual fecal samples clustered together in only 11 out of 37 subjects (one distinct cluster per subject; six placebo subjects and

five GOS subjects; data not shown). The subjects whose samples clustered together had an intra-individual temporal similarity value of greater than 75%. All the other subjects had lower intra-individual temporal stability similarity values. The fecal lactobacilli population was temporally unstable (mean similarity 59.1 ± 25.6%) after GOS-yoghurt consumption and temporally rather unstable (mean similarity 67.2 ± 28.3%) after placebo consumption. However, the difference in temporal stability between the study groups was not significant (P=0.19), due to high variation between the individuals. Since the ingested *L. acidophilus* produced only a single band in the lactobacilli DGGE-profile, it did not constitute a major change of the similarity values between the samples. Lactobacilli-populations have been shown to be temporally unstable in adults[58]. The number of amplicons after GOS- and placebo-yoghurt consumption was similar to the number of amplicons at the baseline.

A composite data set containing all the different DGGE analyses performed was created in the BioNumerics software. According to the PCA plot, none of the sample groups (GOS, placebo, baseline) was distinguishable from the other groups (Figure 13). However, the group of GOS samples was more concise (the samples more similar to each other) than the other sample groups (Figure 13). The group of baseline samples was the most diverse (biggest ellipse; Figure 13). Significant differences were not found in discriminant analysis or MANOVA on temporal stability and bacterial diversity of the composite data sets.

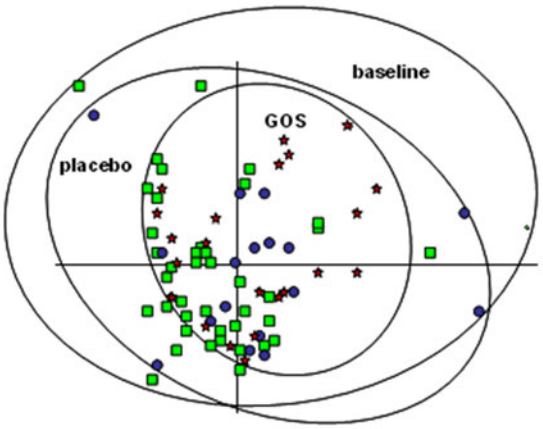

Figure 13. A PCA plot of the composite DGGE data set (containing predominant bacterial, Erec-group, *Lactobacillus*-group, and bifidobacteria specific DGGE analyses) of baseline samples (green squares), samples after galacto-oligosaccharide (GOS) intervention (red stars), and samples after placebo intervention (blue dots).

4.4 Confounding factors in the DNA-based studies of fecal samples (Articles IV and V)

During the past ten years, there have been a wealth of studies in which the relationship between the human gut microbiota and human health has been investigated. Recently there have been several human health-related microbiota studies with partly contradictory results regarding obesity-related microbiota and bifidobacterial abundance of infant microbiota. Since it is probable 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 the most prevalent human gut microbial group composition, diversity, and numbers. The effects of various storage temperatures on the fecal microbiota quantity and composition have been studied previously. However, the results are partly contradictory; some show no differences between the different storage conditions[280,622], whereas in others differences have been found[280,306-307]. Moreover, since 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 a too rigorous DNA-extraction method is used it may result in detecting lower numbers and diversity of Bacteroidetes species. With Firmicutes, however, more rigorous DNA-extraction methods are needed, especially when the matrix is as complex as human feces. Even though there are several studies in which the effectiveness of various DNA-extraction protocols has been assessed[233,235-236,244,246,308], none of them had combined the effects of both storage conditions and DNA-extractions on the diversity, composition, and numbers of the predominant microbiota.

Besides the possible bias caused by different types of sample handling, we also assessed whether the indigenous microbiota itself may cause a bias in the fecal DNA-based experiments. Both ends of the oro-gastrointestinal tract of humans have an abundant microbiota dominated by anaerobically growing bacteria[624]. The number of bacteria in the oral cavity is approximately 10^{11} per gram (wet weight) of dental plaque and 10^{8}–10^{9} per ml of saliva (culturable bacteria[34]), whereas in feces the corresponding figure is 6–9 x 10^{10} per gram (wet weight) (detected with molecular techniques[624]). When molecular techniques are applied to study microbial communities of the GI-tract, it should be kept in mind that the human oro-GI-tract is an open system – i.e. oral bacteria may also be detected with molecular techniques from feces. Although the same bacterial genera can be found in oral and colonic samples, there are only a few studies in which the similarity between oral cavity microbiota and fecal microbiota from the same individuals has been investigated[27].

4.4.1 The effect of sample storage and DNA-extraction on the fecal microbiota results

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[236]. The clostridial populations, namely *E. rectale – B. coccoides* (Erec) group and *C. leptum* (Clept) group were significantly affected both in composition and numbers by the used DNA-extraction protocol. The numbers of both clostridial groups were significantly lower when enzymatic DNA-extraction kit was used as compared to the mechanical DNA-extraction kit. In addition, the composition of Erec-group and Clept-group were different when different commercial kits were applied to the same samples.

The highest numbers of most of the studied bacterial groups were detected from fresh samples and from samples stored at -70^{o}C, however, the differences were not significant for any other group than *Bacteroides* spp. 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. An intriguing finding was also that the highest numbers of *Atopobium* group were obtained after initial storage at 4^{o}C.

The use of different mechanical lysis methods (DNA-extraction protocols 2–8; Figure 1, Article V) did not affect the DNA yield of fresh samples, whereas with stored samples both the kit and different modifications to the kits caused great variations in the DNA yield. The highest DNA yield was achieved with the most rigorous mechanical lysis (DNA-extraction protocol 8; 3x60s; 6.5 m/s) at all storage temperatures, i.e. 4^{o}C, -20^{o}C, and -70^{o}C. The DNA yield was approximately 60% after gentle mechanical lysis (60s 4.5 m/s; $p<0.05$) as compared to the rigorous DNA-extraction, and only 20% after enzymatic lysis ($p<0.05$). 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 storage conditions had a significant effect on the diversity of the predominant fecal bacteria of the studied subjects. Using mechanical DNA-extraction, the predominant bacterial diversity of fresh samples and samples stored at -70^{o}C was significantly higher ($p<0.05$) than that of those stored initially at -20^{o}C or at 4^{o}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 (8–13 amplicons; $p<0.005$) 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 (60s lysis) DNA-extraction protocol was used as compared to the more rigorous mechanical lysis (3x60s). 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 used PCR primers (i.e. V_6-V_8 region vs. V_3-V_5 region), secondarily according to the used DNA-extraction kit (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 the V_6-V_8 region (Figure 2, Article V) and 48–54% after PCR of the V_3-V_5 region (Figure 2, Article V), whereas the similarity of the same samples between different storage conditions was 89–98% when a 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 qPCR (0.5–1 log unit reduction at 4°C and -20°C), but the difference between the quantity of predominant bacteria after DNA-extraction using different DNA-extraction kits was significant at all storage temperatures ($p<0.005$; Figure 3; Article V).

After clustering of the Erec-group DGGE-profiles of both subjects after all different storage – DNA-extraction – PCR combinations, the samples clustered in three major clusters: cluster 1) subject 1, enzymatic DNA-extraction; cluster 2) subject 2, enzymatic DNA-extraction; and cluster 3) subjects 1 and 2, mechanical DNA-extraction. 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% (Figure 2, Article V), whereas the similarity of the same sample after different storage conditions was > 95% when a similar DNA-extraction protocol was applied. Clone library analysis confirmed the different bacterial compositions detected with DGGE (Table 1; Article V). 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 remained about the same regardless of the DNA-extraction protocol applied, as detected with both DGGE and clone libraries (Figure S5, Article V). The initial storage at -20°C or 4°C caused a reduction of ~0.5 log as detected with qPCR when similar protocols were compared. However, the difference was not statistically significant. Moreover, the numbers of Erec-group bacteria were significantly higher after mechanical DNA-extraction ($p<0.05$; ~2 log unit difference; Figure 3, Article V) than after enzymatic DNA-extraction, regardless of the protocol and storage conditions applied.

The samples clustered primarily according to the individual, secondarily (within the primary clusters) according to the used DNA-extraction kit (regardless of modifications) and thirdly according to the storage conditions after the Clept-group DGGE. The similarity of the same samples obtained after DNA-extraction with different kits (in the same storage condition) was 80–87% (Figure 2, Article V),

whereas the similarity of the same sample after different storage conditions was > 94% when similar DNA-extraction protocols were applied, except for sub-samples stored initially at 4°C for two days. The similarity of samples stored at 4°C was 84–93% as compared to the same samples stored at -20°C or at -70°C using identical DNA-extraction protocols. Clone library analysis confirmed the partly different bacterial compositions detected with DGGE (Table 3, Article V). 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 (Figure S5, Article V). The storage conditions did not have a significant effect (~0.5 log reduction at 4°C and -20°C) 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 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 significantly improved DNA extraction efficiency of Clept-group by mechanical DNA-extraction has also been noted previously[236].

The storage conditions and different DNA-extraction methods had no 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 one week's storage at -20°C the numbers of *Bacteroides* spp. were significantly lower (1.6 to 2.5 log reduction, depending on the DNA-extraction modification) than those in fresh samples. The storage at -70°C or initially at 4°C resulted in a non-significant reduction of 0.01–1.5 log units, depending on the DNA-extraction modification. Most of the obesity-related studies that have obtained similar results, i.e. that the relative proportion of Bacteroidetes decreases and the relative proportion of Firmicutes increases in obese individuals[82], have used QIAmp DNA Stool Mini Kit[558-560,562], 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[563-565] or the samples have been studied with fluorescent *in situ* hybridization[378,381,561,564]. Since 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 seems that the contradictory results may be caused, at least partly, by different DNA-extraction protocols.

The different storage conditions and DNA-extraction methods did not affect the bifidobacterial diversity or the composition as detected with bifidobacteria specific DGGE. The storage conditions did not have a significant effect on the numbers of bifidobacteria when similar protocols were compared. Interestingly, however, the lowest numbers of bifidobacteria were found in the fresh samples, contrary to e.g. *Bacteroides* spp. The numbers of bifidobacteria were significantly

higher ($p<0.05$; ~3 log unit difference; Figure 3, Article V) after mechanical DNA-extraction than after enzymatic DNA-extraction as detected with qPCR. A recent example of contradictory findings has involved the bifidobacterial populations of baby feces. Bifidobacterial populations were found to constitute a dominant part of baby feces already decades ago using culture-based methods[143,337]. However, with molecular techniques the results have been partly contradictory. There are numerous studies conducted with molecular techniques in which bifidobacteria have been shown to dominate baby fecal microbiota[225,325,333-334], but there are also some in which bifidobacteria have been found to constitute only a minor part of the infant microbiota[311]. Similarly to the obesity issue, this inconsistency may also result from the different DNA-extraction techniques applied. In those molecular studies in which bifidobacteria have been shown to predominate in the baby feces, mechanical DNA-extraction has been applied[325,334] or the samples have been studied with FISH[225,333]. In those studies in which 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 our study has been applied[311]. Since 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 probably explain these contradictory results. In addition, Nakamura *et al.*[310] showed that when enzymatic DNA-extraction was applied bifidobacterial the 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, it has previously been found[236] that with more rigorous mechanical disruption the proportion of Actinobacteria (e.g. bifidobacteria and *Atopobium* group) increased. Interestingly, the protocol which yielded the lowest levels of Actinobacteria was based on the same kit that we used for enzymatic DNA-extraction[236]. However, we used the kit according to the manufacturer's instructions, whereas Salonen *et al.*[236] added 3x30 s bead beating and extended the duration of heat lysis in their study. Even with these modifications the proportion of Actinobacteria was low.

The diversity and composition of *Lactobacillus* group was 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 the 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; Figure 3, Article V) in all evaluated storage conditions. Unexpectedly, the numbers of *Atopobium* group bacteria were significantly higher ($p<0.05$; > 1 log unit difference) after initial storage at -20°C for one week or at 4°C for two days than those from fresh samples or samples stored solely at -70°C. 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 two days and thereafter at -70°C and when DNA-extraction was performed mechanically (3 x 60s), 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. (part of the *Atopobium* group) cell wall contains a unique A4β-type peptidoglycan[152]. Therefore it may be 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 human predominant microbiota[55], 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[63-64,77,251]. Moreover, the possible underestimation of *Atopobium* group bacteria, and Actinobacteria in general have also been reported elsewhere[236,309].

4.4.2 Salivary microbiota vs. fecal microbiota

Oral cavity contains different micro-environments (cheeks, palate, tongue, tooth surfaces, gingival areas, and saliva) with their own microbiota[18]. Salivary microbiota reflects a mixture of bacteria washed off from the various surfaces, especially from the tongue[34]. A large diversity of bacteria has been detected in the oral cavity, including low GC Gram positives (e.g. streptococci and clostridia-group), fusobacteria, actinobacteria, different proteobacteria, *Prevotella, Porphyromonas, Bacteroides,* and spirochetes. Members of the Clostridial cluster XIVa have occasionally been detected in oral samples[22]. In the oral cavity both bifidobacteria and lactobacilli can be detected, but their occurrence is reversed compared to feces: lactobacilli are a common finding in the oral cavity, whereas bifidobacteria are less frequently detected.

The aim was to investigate whether similar features concerning the diversity and temporal stability of the predominant microbiota and selected bacterial groups – namely the Clostridial cluster XIVa, bifidobacteria and lactobacilli – could be detected intra-individually in saliva and feces. Special focus was put on the species distribution of the genera *Lactobacillus* and *Bifidobacterium* at these two sites. The subjects consumed a commercial probiotic capsule preparation (Trevis, 54 capsules; Ipex Medical) according to the manufacturer's instructions (three capsules each day) for 2 weeks. Trevis capsules contain a mixture of *Lactobacillus acidophilus* LaCH-5, *Bifidobacterium animalis* subsp. *lactis* Bb-12 and yoghurt starter bacteria, totaling 10^9–10^{10} organisms per capsule.

Compliance was evaluated with culture-based techniques and DGGE analysis in this study. Probiotic strains were not found in any of the baseline samples, whereas RAPD types identical to *B. animalis* subsp. *lactis* Bb12 were found in all fecal samples during probiotic ingestion. Ingested probiotic strains were not found in any of our salivary samples.

DGGE analysis targeted to the predominant microbiota showed intra-individual diversity as well as uniqueness of both fecal and salivary microbiota (Figure 1A, Article IV). When all the samples were clustered together, salivary samples formed one cluster and fecal samples another. Both sample-type clusters had further

individual-based subclusters. The DGGE-profiles of fecal and salivary samples differed substantially (mean similarity 24 ± 7%). The predominant fecal microbiota was temporally relatively stable (mean similarity 83 ± 5%) and the salivary microbiota mostly stable (mean similarity 92 ± 3%) during the study period. These findings are consistent with previous studies in which it has been showed that the fecal and salivary microbiota are host specific and temporally relatively stable[56,58,242,625]. We found a significant difference between the number of amplicons detected in the fecal and salivary profiles (feces > saliva; $p<0.05$). Since Aas *et al*.[18] found 20–30 different predominant species in most oral sites, our predominant salivary bacterial DGGE results are in accordance with this previous study.

DGGE analysis targeted to the Erec-group showed intra-individual diversity as well as uniqueness of both fecal and salivary microbiota (Figure 1B, Article IV). Erec-profiles of fecal samples clustered together according to the person, whereas in salivary samples individual clustering was noticed in eight out of ten individuals. The similarity between fecal and salivary samples from the same individual at a given time point was very low or non-existent (mean similarity 18 ± 11%). All fecal samples clustered together as one distinct cluster and all salivary samples as another. In an earlier study of this thesis (Article II) it was shown that each band position in DGGE-gels contained only one phylotype. We may therefore assume that the phylotypes present in the feces and saliva are mostly different. Erec-group bacteria were temporally stable in most cases (mean similarity: feces 94 ± 3%, saliva 91 ± 7%) during the study period, as has been described previously[242].The Erec-group diversity in salivary samples of this study was significantly lower than in the fecal samples ($p<0.05$), although 3–12 amplicons were found in each sample, indicating that the diversity of the Erec-group in saliva in some subjects may be slightly greater than has previously been shown with other techniques[22,626].

The fecal and salivary *Lactobacillus*-group DGGE-profiles of this study were more similar to each other (mean similarity 59 ± 11%) than the feces- and saliva-derived profiles of the other studied bacterial groups. In addition, fecal *Lactobacillus*-group profiles of nearly all the people were rather unstable (mean similarity 69 ± 7%; Figure 1C, Article IV), whereas salivary *Lactobacillus*-group profiles remained rather stable (mean similarity 90 ± 8%). Based on the comparisons made between different sampling points (baseline without probiotic consumption vs. samples during probiotic consumption), the ingested *L. acidophilus* LaCH-5 strain, which was seen in the fecal lactobacilli DGGE-profile during probiotic ingestion, does not alone explain the instability. Since the LaCH-5 strain produced only a single band, it did not constitute a major change in the similarity values between the samples. In the salivary samples there were no amplicons migrating identically to *L. acidophilus* LaCH-5. In BioNumerics analysis the lack of stable and host-specific *Lactobacillus*-group population resulted in a complete lack of subject grouping in fecal samples and sample type grouping (feces vs. saliva). The lack of subject grouping has also been reported previously for fecal samples[58]. The number of amplicons detected in our study was higher in fecal samples than in salivary samples.

Bifidobacterial DGGE-profiles of fecal samples of a given subject clustered together in nine out of ten people in this study. In these cases the fecal bifidobacte-

rial population remained temporally rather stable (Figure 1D, Article IV), as has also been reported previously[256]. Only approximately half of the salivary samples (17/30 samples) gave a positive PCR-result, although several additional variations of the PCR-protocol were tested. The salivary bifidobacterial populations were temporally stable in three subjects and unstable in one subject. Of the remaining six subjects, only one sample from three subjects and two samples from another subject produced a PCR product – thus not affording temporal stability to be determined. The bifidobacterial profiles did not cluster according to the sample type, even though the similarity between fecal samples and salivary samples collected at the same time was low. The fecal samples contained significantly more amplicons than the salivary samples at all sampling time points ($p<0.05$). Since the PCR-primers, which were used for the DGGE-based diversity assessment, did not fully target (1 mismatch) the ingested *B. animalis* subsp. *lactis* Bb12[610], this strain was not seen in the bifidobacterial DGGE-profiles, and therefore did not affect the temporal stability of the fecal and salivary profiles. However, Bb12 was detected in fecal samples from 7 of the 10 subjects using *B. animalis* subsp. *lactis* Bb12 targeting DGGE. There were no amplicons migrating identically to *B. animalis* subsp. *lactis* Bb-12 in any of the salivary samples.

The number of culturable bacteria remained stable during the probiotic consumption in both fecal and salivary samples. The number of culturable anaerobic bacteria was significantly higher in feces (mean log 10.5 cfu/g) than in saliva (mean log 8.3 cfu/g) ($p<0.05$), whereas the number of culturable aerobic bacteria was significantly higher in saliva (mean log 7.8 cfu/g) than in feces (mean log 7.1 cfu/g) ($p<0.05$). Mean numbers of culturable bacteria on media without tetracycline at different sampling occasions are shown in Figure 2 of Article IV. The numbers of both bifidobacteria and lactobacilli were significantly higher in feces as compared to saliva ($p<0.05$) (Fig. 2). The numbers of culturable bacteria found in salivary and fecal samples were in accordance with previous studies[145, 372,627].

Isolates with an identical RAPD type to the ingested *B. animalis* subsp. *lactis* Bb-12 strain were recovered from fecal samples but not from the salivary samples of all subjects during probiotic consumption, whereas isolates of *L. acidophilus* LaCH-5 strain were recovered from fecal samples of all the subjects after 1 week of probiotic consumption and from nine out of ten subjects after 2 weeks.

From each subject 3–6 indigenous salivary lactobacilli RAPD types and 1–5 indigenous fecal lactobacilli RAPD types were detected (Table 3; Article IV). In addition, identical indigenous lactobacilli RAPD types were found in saliva and feces for eight of the ten subjects (Table 3; Article IV). These included *L. rhamnosus*, *L. gasseri*, *L. paracasei*, *L. plantarum* group (comprising *L. plantarum*, *L. arizonensis,* and *L. pentosus*), and a *Lactobacillus* sp. In a study of Dal Bello & Hertel[27], *Lactobacillus*-populations of salivary and oral samples of three subjects were compared by culture and RAPD typing in addition to DGGE. They found that *L. gasseri*, *L. paracasei*, *L. rhamnosus,* and *L. vaginalis* were most commonly detected among the predominant lactobacilli in the saliva and feces of their three subjects. However, we found *L. vaginalis* only from one subject's saliva and from none of the fecal samples of our subjects. *L. gasseri*, *L. paracasei*, and *L. rhamnosus* were

commonly detected from our subjects. The similarity of the fecal and salivary *Lactobacillus* populations found in this and another study[27] indicate that at least some *Lactobacillus* species/strains are able to live in different niches in the human orogastrointestinal tract – although the oral cavity and colon differ in several aspects including redox potential, nutrients, mucosal surfaces, and co-existing members of the specific microbial community.

Between 2 and 8 indigenous RAPD types were found per subject from fecal isolates, and 2 to 9 per subject from salivary isolates from Beerens agar. However, only 1–7 fecal and 0–2 salivary RAPD types per subject were confirmed to be bifidobacteria (after bifidobacteria-specific PCR[256]), indicating that most salivary biotypes from the Beerens agar were not bifidobacteria. By contrast, 80% (37/46) of the fecal biotypes from the Beerens agar were bifidobacteria. No identical indigenous RAPD types were found between saliva and feces. After sequencing of the different bifidobacteria RAPD types, multiple indigenous bifidobacterial genotypes, including *B. longum*, *B. adolescentis*, *B. catenulatum/pseudocatenulatum*, *B. bifidum*, and *B. angulatum* were detected in feces within an individual, whereas only *B. dentium* and *B. bifidum* were detected from saliva. We found *B. dentium* from the saliva of five subjects. *B. dentium* was not found in fecal samples, which is consistent with one published culture-based analysis[142]. However, it has been found in feces in DNA-based studies[145]. We used culture-based identification in the present study, since we wanted to determine whether the bifidobacteria detected from the feces were alive and therefore most likely to represent autochthonous strains. The levels of *B. dentium* in our salivary samples were $2 \cdot 10^5$ cfu/ml (mean), which accounts for 10^6–10^8 cells per 1000 ml saliva – which is the average quantity of saliva ingested daily[34]. Therefore the *B. dentium* strains found in the fecal samples by DNA-based methods may originate from saliva and actually be allochthonous.

5. Summary and conclusions

The first hypothesis was that the developed molecular tools enable rapid and cost-effective characterization of the human predominant fecal microbiota. As shown in Articles I, II, and V, all the developed methods (rRNA-based predominant bacterial DGGE, rRNA- and DNA-based *Eubacterium rectale – Blautia coccoides* group (Erec) DGGE, DNA-based *C. leptum* group (Clept) DGGE, DNA-based *Bacteroides* spp. DGGE, and qPCR methods targeting predominant bacteria, Erec-group, Clept-group, *Bacteroides* spp., bifidobacteria and *Atopobium* group) proved to be sensitive and highly specific. All the developed methods enabled rapid and cost-effective characterization of the human fecal and salivary predominant microbiota. Predominant bacteria, Erec-group, Clept-group, and *Bacteroides* spp., populations of healthy adults proved to be temporally rather stable showing intra-individual diversity and inter-individual variability. Furthermore, the rRNA-based profiles showed more temporal instability than DNA-based profiles of predominant bacteria and Erec-group bacteria.

The second hypothesis was that the fecal microbiota of people having IBS differs from that of healthy adults, and that the difference is distinguishable with molecular microbiological techniques. According to the results, the studied clostridial groups (Erec, Clept, *C. lituseburense,* and *C. histolyticum*) represented the dominant fecal microbiota of most of the studied subjects, comprising altogether 29–87% of the total bacteria as determined by the hybridized 16S rRNA of TRAC-analysis. Erec-group was the dominant subgroup of clostridia, accounting on average for 43% of total bacteria in control subjects and 30% and 50% in irritable bowel syndrome (IBS) subjects affected by constipation and diarrhea, respectively. The proportion of Erec-group was significantly lower in the constipation type IBS subjects than in the healthy controls. DNA-based and rRNA-based DGGE analyses targeted to the predominant bacterial population showed considerable biodiversity as well as uniqueness of the microbiota in each subject in both control and IBS subject groups. The rRNA-based DGGE profiles of the fecal microbiota of the IBS subjects further indicated higher temporal instability of their microbiota composition as compared to the control subjects. Our observations indicate that in addition to instability of fecal active predominant bacterial population, clostridial microbiota may also be involved in IBS.

The third hypothesis was that the fecal microbiota of elderly people differs from that of younger adults and that the elderly fecal microbiota may be modified with a

GOS-yoghurt intervention. We did not find any differences between elderly and younger adults in temporal stability of the studied bacterial populations (predominant bacteria, Erec-group, bifidobacteria, and lactobacilli). However, the difference in diversity of predominant bacterial population and Erec-group bacteria was found to be significantly higher in elderly subjects as compared to younger adults. Consumption of probiotic yoghurt containing galacto-oligosaccharide (GOS) for three weeks did not significantly affect the diversity or temporal stability of predominant bacterial, bifidobacterial, *Lactobacillus*-group or Erec-group fecal populations in elderly subjects. On average, the Erec-group bacteria remained stable, the predominant bacterial and bifidobacterial populations remained reasonably stable and the *Lactobacillus*-group was unstable in both study groups. However, the composite data set containing all DGGE analyses of the study showed that the microbial communities from the GOS-supplemented subjects were more similar to each other than those of the control subjects. Therefore, the GOS-yoghurt may have a stabilizing effect on the predominant fecal microbiota of elderly people.

The fourth hypothesis was that there might be confounding factors responsible for the contradictory results obtained in many recent studies – especially differences in storage conditions and DNA-extraction protocols of fecal samples. According to the results, the DNA-extraction did not affect the diversity, composition, or quantity of *Bacteroides* spp., whereas after one week's storage at -20^{o}C the numbers of *Bacteroides* spp. were 1.6–2.5 log-units lower ($p<0.05$). Furthermore, the numbers of predominant bacteria, Erec-group, Clept-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 leads to the detection of higher bacterial numbers and higher bacterial population diversity in human fecal samples. Since 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.

Another part of the hypothesis was whether the microbiota of the upper gastrointestinal tract may also influence the results obtained from fecal samples with DNA-based methods, since the gastrointestinal tract is an open system. Therefore the similarity of the salivary and fecal microbiota was studied. The predominant bacteria, bifidobacteria, and Erec-group bacteria of the oral cavity and feces were generally stable during probiotic consumption, showing more diversity in feces than in saliva and different species compositions for the two sampling sites. By contrast lactobacilli, which are known to inhabit several ecological niches, showed temporal instability in both feces and saliva. Furthermore, fecal and salivary samples contained identical indigenous *Lactobacillus* genotypes (*L. rhamnosus*, *L. gasseri*, *L. paracasei*, *L. plantarum* group, and a *Lactobacillus* sp.) in most subjects.

6. Future outlook

In 1959 Jordan concluded with regard to problems related to gut microbiota studies: "Whenever a man gets the idea that he is going to work out the bacteriology of the intestinal tract of any mammal, the time has come to have him quietly removed to some suitable institution"[341]. A lot has happened in the field of gastrointestinal microbiota research since those days. Especially after the initiation of the Pro EU Health cluster in the beginning of 21st century and the Human Microbiome Project (HMP) in the U.S. and MetaHit in Europe in recent years, the knowledge of GIT microbiota has greatly advanced. However, occasionally it seems that the culture-based knowledge obtained in the 1960s and 1970s has been forgotten, or regarded as "old-fashioned" or even "out-dated". Moreover, it appears that the used method is of more importance, than the study design and which method – however "out-dated" – would provide the best possibility to answer the question proposed.

The recent large projects have created vast amounts of sequence data that many times correspond to "uncultured bacterial clones" or genes coding for "unknown functions". In order to fully exploit the enormous databanks hitherto created, culture-based studies would be needed for the isolation of the currently "uncultured bacteria". Besides using culture-based approaches for the identification of "uncultured bacteria" and determination of their metabolic properties, single-cell genomics together with flow cytometry sorting and/or (microscopic) optical tweezers would enable the genome analysis of a single bacterium, thus minimizing the possible misinterpretations of giant data sets created with the metagenomic approach. Besides assessing the theoretical metabolic properties obtained with DNA-based metagenomic analysis, more information would be needed on the actual activity of the complex GIT microbial population. Moreover, the combination of proteomics, transcriptomics, and metabolomics would shed more light on the host response.

Another recent hot topic in the field of GIT microbiota research has been the correlation of certain bacteria/bacterial groups with certain diseases/disorders. Nowadays it appears to be easier to list the diseases/disorders that have not been associated with disturbances in the GIT microbiota, than those that have. However, correlation does not equal causality. In order to actually authenticate the relationship between diseases and gut microbiota, large longitudinal population cohorts with all the background information (including diet, medical history, genetics etc.) would be needed.

In conclusion, although a large amount of data has been generated during the past ten years in the field of GIT microbiota research, many questions still remain unanswered. Therefore, besides having all the relevant background information and numerous samples, more interdisciplinary research across microbiology, immunology, genetics, epigenetics, proteomics, transcriptomics, metabolomics, and physiology would be needed.

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ARTICLE I

Prevalence and temporal stability of selected clostridial groups in irritable bowel syndrome in relation to predominant faecal bacteria

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Prevalence and temporal stability of selected clostridial groups in irritable bowel syndrome in relation to predominant faecal bacteria

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The differences in faecal bacterial population between irritable bowel syndrome (IBS) and control subjects have been reported in several studies. The aim of the present study was to compare the predominant and clostridial faecal microbiota of IBS subjects and healthy controls by applying denaturing gradient gel electrophoresis (DGGE) and a recently developed multiplexed and quantitative hybridization-based technique, transcript analysis with the aid of affinity capture (TRAC). According to the results, the studied clostridial groups (*Clostridium histolyticum*, *Clostridium coccoides-Eubacterium rectale*, *Clostridium lituseburense* and *Clostridium leptum*) represented the dominant faecal microbiota of most of the studied subjects, comprising altogether 29–87 % of the total bacteria as determined by the hybridized 16S rRNA. The *C. coccoides-E. rectale* group was the dominant subgroup of clostridia, contributing a mean of 43 % of the total bacteria in control subjects and 30 % (constipation type) to 50 % (diarrhoea type) in different IBS symptom category subjects. The proportion of the *C. coccoides-E. rectale* group was found to be significantly lower in the constipation-type IBS subjects than in the control subjects. DNA-based PCR-DGGE and RNA-based RT-PCR-DGGE analyses targeted to the predominant bacterial population showed considerable biodiversity as well as uniqueness of the microbiota in each subject, in both control and IBS subject groups. The RT-PCR-DGGE profiles of the IBS subjects further indicated higher instability of the bacterial population compared to the control subjects. The observations suggest that clostridial microbiota, in addition to the instability of the active predominant faecal bacterial population, may be involved in IBS.

INTRODUCTION

The composition of the resident intestinal microbiota varies between individuals, and the predominant population is fairly stable under normal conditions (Zoetendal *et al.*, 1998; Harmsen *et al.*, 2002a; Vanhoutte *et al.*, 2004; Mättö *et al.*, 2005). However, several factors, such as antibiotic therapy, ageing and disease, may cause disturbances in the intestinal balance. Transient disturbance of the intestinal microbiota during antibiotic therapy has been shown in several studies (Edlund & Nord, 2000; Donskey *et al.*, 2003). Changes in the intestinal microbiota have also been suggested to occur in certain intestinal diseases and disorders, such as inflammatory bowel disease (IBD) (Seksik *et al.*, 2003) and irritable bowel syndrome (IBS) (Madden & Hunter, 2002). IBS is an intestinal disorder that involves continuous or recurrent intestinal pain or discomfort that is relieved during defecation. In addition, IBS symptoms include bloating, altered stool frequency, form or passage, and passage of mucus (Thompson *et al.*, 1999). The existence of abnormal colonic fermentation in IBS (King *et al.*, 1998) and alleviation of IBS symptoms by eradication of small intestinal bacterial overgrowth by antibiotic therapy (Pimentel *et al.*, 2000), suggest that the intestinal microbiota has a role in IBS. Some studies have reported differences in the faecal bacterial population between IBS and control subjects (Balsari *et al.*, 1982; Bradley *et al.*, 1987; Madden & Hunter, 2002; Mättö *et al.*, 2005; Malinen *et al.*, 2005). However, the results obtained in earlier studies are partly contradictory, and with the exception of those of Mättö *et al.* (2005) and Malinen *et al.* (2005), are based on the culture-based analysis of the microbiota. Therefore, the role of intestinal microbiota in IBS is still poorly known. Since IBS is often linked with extensive gas production in the colon (King *et al.*, 1998), and some bacterial groups are more prone to gas production than others (Cato *et al.*, 1986), the possible role of the composition and/or activity of clostridia and related bacteria in intestinal (im)balance warrants further study.

Abbreviations: DGGE, denaturing gradient gel electrophoresis; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; TRAC, transcript analysis with the aid of affinity capture.

The aim of the present study was to compare the diversity and temporal stability of the predominant faecal microbiota, with both DNA- and RNA-based denaturing gradient gel electrophoresis (DGGE), to reveal possible differences between IBS subjects and healthy controls. In addition, the composition, abundance and stability of selected clostridial groups were studied to further assess possible differences.

METHODS

Samples. Five male and 11 female subjects were included in the IBS subject group (age 24–64 years; mean 45 years). The IBS group consisted of subjects with diarrhoea-dominant (seven subjects), constipation-dominant (six subjects) and alternating (mixed) type (three subjects) symptoms. The control subject group consisted of four male and 12 female subjects that were 26–63 years of age (mean 45 years). The exclusion criterion for both groups was antimicrobial therapy during the 2 months prior to each sampling timepoint. Faecal samples from the 32 subjects were obtained on two occasions 6 months apart (0 and 6 months). More detailed information about the IBS and control subject groups, as well as sampling and sample handling, has been described previously by Mättö *et al.* (2005).

DNA extraction. DNA was extracted from 300 mg of faecal material using the FastDNA Spin Kit for Soil (QBIOgene) according to manufacturer's instructions, with the modification that the bacterial cells were broken with a Fast Prep instrument at 6·0 m s^{-1} for 60 s three times. The isolated DNA was stored at −20 °C until examined.

RNA extraction and biotinylation. RNA was extracted from faeces as described elsewhere (Zoetendal *et al.*, 1998) with minor modifications (Satokari *et al.*, 2005), and further purified by using the clean-up protocol of the RNeasy mini kit (Qiagen). Total RNA was quantified by A_{260} measurement, and the purity of RNA was evaluated by agarose gel electrophoresis. Purified RNA used for quantitative analysis (see below) was biotinylated by using Photoprobe biotin (Vector Laboratories). Biotinylation was performed by exposing the RNA to long-wave UV light (365 nm) for 30 min, and the subsequent purification of the RNA from free biotin was performed according to the manufacturer's instructions, with slight modifications according to Satokari *et al.* (2005).

Specific quantification of 16S rRNA. Multiplexed quantification of clostridial 16S rRNA was performed with the transcript analysis with the aid of affinity capture (TRAC) technique, according to Satokari *et al.* (2005). Specific 16S rRNA-targeted probes for different groups of clostridia and a universal bacterial probe were used in the study (Table 1). The probes were labelled with 6-FAM (6-carboxyfluorescein) at the 5′ end and HPLC-purified. Briefly, the TRAC analysis was performed as follows: RNA (maximum amount 5–10 ng) was denatured at 70 °C for 2 min and hybridized with all the oligonucleotide probes at 50 °C for 1 h. After hybridization, the biotin–nucleic acid probe complexes were captured on magnetic streptavidin-coated microparticles and washed. The hybridized probes were eluted, and their identity and quantity were determined by capillary electrophoresis with an ABI PRISM 3100 genetic analyser. The signal intensities of the recorded probes corresponded to the amount of target nucleic acid in the mixture, while the size indicated the target. Results were expressed as the percentage of total bacterial 16S rRNA that was detected by the Bact338-IIA probe, and as the mean ± SD of triplicate measurements.

PCR and RT-PCR. Partial 16S rRNA genes were PCR-amplified using primers U968-f+GC and U1401-r (Table 1), as described by Mättö *et al.* (2005). RT-PCR was performed with the Qiagen OneStep RT-PCR kit according to the manufacturer's instructions, with or without a Q-solution. Primers used for the RT-PCR were the same as those used for the PCR (Table 1). The PCR programme consisted of reverse transcription performed at 50 °C for 30 min,

Table 1. Probes and primers used

Target group	Short name	Probe/primer	Sequence (5′→3′)	Reference
C. lituseburense group*	Clit group	Clit135¶	GTTATCCGTGTGTACAGGG	Franks *et al.* (1998)
C. histolyticum group†	Chis group	Chis150¶	TTATGCGGTATTAATCTYCCTTT	Franks *et al.* (1998)
C. coccoides-E. rectale group‡	Erec group	Erec482-5A¶#	GCTTCTTAGTCARGTACCGAAAAA	Franks *et al.* (1998); Satokari *et al.* (2005)
C. leptum group§	Clept group	Clept1240¶	GTTTTRTCAACGGCAGTC	Sghir *et al.* (2000)
Bacteria		Bact338-IIA¶**	GCTGCCTCCCGTAGGAGTIIA	Amann *et al.* (1990); Satokari *et al.* (2005)
Bacteria‖		U968-f +GC††	CGCCCGGGGCGCGCCCCGGGCGG-GGCGGGGGCACGGGGGGAACGC-GAAGAACCTTA	Nübel *et al.* (1996)
Bacteria‖		U1401-r††	CGG TGT GTA CAA GAC CC	Nübel *et al.* (1996)

*Clostridial phylogenetic cluster XI (Collins *et al.*, 1994).
†Clostridial phylogenetic clusters I and II (Collins *et al.*, 1994).
‡Clostridial phylogenetic clusters XIVa and XIVb (Collins *et al.*, 1994).
§Clostridial phylogenetic cluster IV (Collins *et al.*, 1994).
‖Partial 16S rRNA gene (V_6–V_8 hypervariable region).
¶Used as a probe in the quantitative TRAC analysis.
#The Erec482 (Franks *et al.*, 1998) probe was tailed with an additional AAAAA sequence (Erec482-5A) at the 3′ end for size-distinction purposes.
**The Bact338 (Amann *et al.*, 1990) probe was tailed with an additional IIA sequence (Bact338-IIA, where I is inosine) at the 3′ end for size-distinction purposes.
††Used as a primer in PCR-DGGE and RT-PCR-DGGE.

followed by denaturation at 95 °C for 15 min. Thereafter, the RT-PCR programme was similar to the PCR programme: 35 cycles of denaturing at 94 °C for 30 s, primer annealing at 50 °C for 20 s and elongation at 72 °C for 40 s, and final extension for 10 min at 72 °C.

DGGE analysis of 16S rDNA fragments. DGGE analysis was performed as described by Mättö *et al.* (2005). The similarity of the PCR-DGGE profiles of the samples obtained from a single subject at different time-points was compared to evaluate the temporal stability of the predominant faecal bacterial population. The comparison of the profiles was performed by visual inspection of the gels by two researchers and by calculating the similarity percentage using BioNumerics software version 3.0 (Applied Maths BVBA). Clustering was performed with Pearson correlation and the unweighted pair group method with arithmetic mean (UPGMA). Amplicons with a total surface area of at least 1 % were included in the similarity analysis. Similarity value thresholds indicating stable, rather stable and unstable DGGE profiles were determined according to the visual inspection. A DGGE profile was defined as stable if profiles at different time-points were identical (had the same amplicons and no/minor intensity differences), rather stable if they were similar (had differences in presence and intensity of few amplicons), and unstable if the profiles were different (had differences in presence and intensity of several amplicons). Similarity value thresholds for RNA-based and DNA-based profiles were determined separately.

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

RESULTS AND DISCUSSION

In this study, the predominant and clostridial faecal microbiota of IBS subjects and healthy controls were compared to reveal possible differences in the composition, abundance and stability of selected groups. Mättö *et al.* (2005) have shown by DNA-based analysis that IBS subjects have more instability in their predominant faecal microbiota than healthy controls. We broadened the study by examining whether the instability which was seen in the DNA-based analysis could also be seen in RNA-based analyses, both predominant microbiota and clostridia-targeted. In addition to stability, the composition and abundance of the selected clostridial groups were studied. Clostridia were selected as a target group in this study, since disturbed metabolism of intestinal gases, as well as increased sensitivity of the colon, has been associated with IBS symptoms (King *et al.*, 1998). Since clostridia are able to produce gas, they may play a role in IBS symptoms (Cato *et al.*, 1986).

Temporal stability of predominant bacterial populations

The temporal intraindividual stability of the predominant bacteria was studied with DNA-based PCR-DGGE and RNA-based RT-PCR-DGGE to determine whether the stability/instability of clostridial populations corresponded with the stability/instability of the predominant bacterial population, and if any differences could be observed between control and IBS subjects (Fig. 1). DNA-based PCR-DGGE and RNA-based RT-PCR-DGGE analyses targeted to the predominant bacterial population showed considerable intraindividual biodiversity, as well as uniqueness of the faecal microbiota in both control and IBS subject groups in this study. PCR-DGGE and RT-PCR-DGGE analysis with universal bacterial primers did not reveal any IBS-specific amplicons. PCR-DGGE and RT-PCR-DGGE profiles were heterogeneous, containing about 20–30 and 15–25 amplicons, respectively. The RT-PCR-DGGE profiles of IBS subjects (16 ± 5 amplicons) and control subjects (16 ± 4 amplicons) contained significantly fewer amplicons than the PCR-DGGE profiles (IBS subjects, 21 ± 5 amplicons; control subjects, 23 ± 5 amplicons) ($P < 0{\cdot}05$; Fig. 1). There were differences in all parts of the denaturing gradient. Some amplicons were more prominent in the RNA-derived profile than in the DNA-derived profile, and vice versa. In addition, some bands were missing from the RNA-derived profiles and some from the DNA-derived profiles. Furthermore, the amplicons with the highest GC content (migrating to the lower part of the DGGE gel) were mostly missing in the RT-PCR-DGGE profiles (Fig. 1). The clear difference between the DNA- and RNA-derived predominant faecal bacterial populations has also been reported by Tannock *et al.* (2004). The differences between the DNA- and RNA-derived profiles may be due to several reasons. The resolving power of RT-PCR is limited by the efficiency of RNA-to-cDNA conversion (Bustin & Nolan, 2004). In addition, DNA obtained from complex samples does not reflect metabolic activity (Josephson *et al.*, 1993), whereas rRNA is regarded as an indicator of total bacterial activity (Felske *et al.*, 1997). Therefore, the amplicons that are missing from

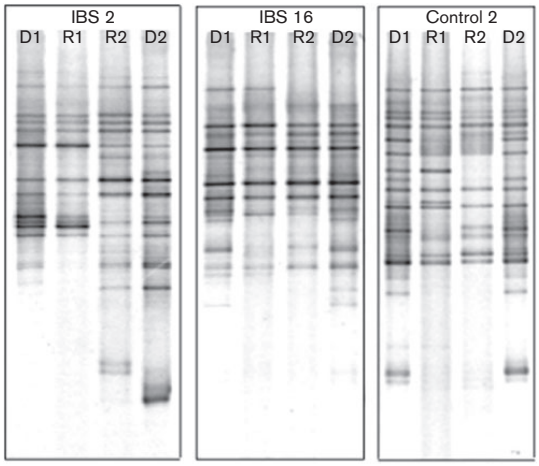

Fig. 1. Comparison of DNA-based PCR-DGGE and RNA-based RT-PCR-DGGE profiles during a 6 month time-period for two IBS subjects and one control subject. Lanes D1 correspond to DNA-based DGGE profiles at baseline, lanes R1 to RNA-based DGGE profiles at baseline, lanes R2 to RNA-based DGGE profiles 6 months later and lanes D2 to DNA-based DGGE profiles 6 months later. The panels are from different individuals with IBS (IBS2 and IBS16) and from a healthy control (Control 2).

the RNA-derived profiles may reflect a low activity in that part of the faecal microbiota.

When DGGE profiles derived from faecal RNA amplicons were compared to the DGGE profiles derived from faecal DNA amplicons in this study, the temporal intraindividual similarity values of RT-PCR-DGGE and PCR-DGGE profiles were significantly higher than the intraindividual similarity values between DNA- and RNA-based DGGE profiles at a given time-point in nearly all cases ($P < 0{\cdot}05$; Table 2). However, since each individual had unique PCR-DGGE and RT-PCR-DGGE profiles, the intraindividual RNA- and DNA-based DGGE profiles resembled each other more than the interindividual RNA-based profiles (Fig. 1). In the present study, the assessment of temporal stability of the predominant microbiota with DNA-based PCR-DGGE did not reveal more instability in the IBS subjects than in the control group subjects (Table 2), contrary to the findings of Mättö *et al.* (2005). However, less similarity (i.e. more instability) was observed in the DGGE profiles derived from faecal RNA amplicons of the IBS subjects than the control subjects (Table 2). In addition, the similarity value range of RNA-derived DGGE profiles of IBS subjects was much larger than that of the control subjects (Table 2). The main difference between the study group of Mättö *et al.* (2005) and the study group in this study was that we did not include subjects who had received antimicrobial therapy during the 2 months prior to the two sampling time-points, since antibiotic therapy can cause disturbances in the intestinal microbiota (Edlund & Nord, 2000; Donskey *et al.*, 2003), and may also affect the predominant bacterial groups (Bartosch *et al.*, 2004). Under normal circumstances, the predominant intestinal microbiota of an adult individual has been reported to be fairly stable (Zoetendal *et al.*, 1998, 2001; Seksik *et al.*, 2003; Vanhoutte *et al.*, 2004). The effect of disease on the stability of intestinal microbiota is clearly seen in the study of Seksik *et al.* (2003), in which a marked instability of the predominant faecal microbiota was observed in four IBD patients. In our study, temporal instability was observed in both IBS and control subjects (with both DNA- and RNA-based DGGE). In previous studies in which the long-term temporal stability of the predominant microbiota from healthy subjects has been assessed, the number of subjects has been limited (one to four individuals) (Zoetendal *et al.*, 1998, 2001; Seksik *et al.*, 2003; Vanhoutte *et al.*, 2004).

The intraindividual similarity values of PCR-DGGE and RT-PCR-DGGE profiles of IBS subjects between time-points 1 (baseline) and 2 (6 months) varied between 45 and 95 %, and between 39 and 95 %, respectively, whereas the similarity values of control subjects varied between 59 and 97 %, and between 68 and 94 %, respectively (Table 2). When IBS patients were divided into subgroups according to their symptoms in our study, the greatest intraindividual variability in DGGE similarity values within the subject group was seen in the diarrhoea-type subgroup and the smallest in the mixed-type subgroup (Table 2). The great intraindividual variation seen in the diarrhoea-type subgroup might be due to different phases of the syndrome. If a patient is experiencing a more symptomatic phase of IBS, the predominant bacterial population might be significantly different to that when symptom-free, in a similar manner to observations for IBD patients (Seksik *et al.*, 2003). However, we did not have data on the symptomatic activity of each IBS subject, so this conclusion remains speculative. Further studies are needed to assess the influence of the more symptomatic phase of the syndrome on the stability of the faecal microbiota. The smallest intraindividual variability in our study, shown by the mixed-type subject group, is at least partly explained by the small number of subjects (three), so further studies are needed with larger patient groups.

Proportion of clostridia

In the present study, the recently developed TRAC technique (Satokari *et al.*, 2005) allowed the multiplexed quantification of the clostridial groups. The sensitivity of the technique allows as little as 0·05 ng of total RNA to be detected, but the quantification of proportions is limited by the dynamic range of measurement. In practice, it is feasible to measure 100–200-fold differences in the amounts of individual target RNAs (Satokari *et al.*, 2005). According to our results, the studied clostridial groups [*Clostridium. histolyticum* (probe Chis150), *Clostridium coccoides-Eubacterium rectale* (probe Erec482), *Clostridium lituseburense* (probe Clit135) and *Clostridium leptum* (probe Clept1240) groups] represented the dominant faecal microbiota in most of the studied subjects (26 out of 32 subjects, including both IBS and control subjects), contributing altogether 29–87 %, as determined by the hybridized 16S rRNA (Table 3). This is in agreement with other studies, where several clostridial groups have been studied from clone libraries of adult faecal samples (Wilson & Blitchington, 1996; Suau *et al.*, 1999; Hayshi *et al.*, 2002; Mangin *et al.*, 2004). The proportion of each studied clostridial group showed marked interindividual variation (Table 3).

There were only small differences in the proportions of the *C. leptum*, *C. lituseburense* and *C. histolyticum* groups between IBS and control subjects (Table 3). It has been reported that the *C. lituseburense* and *C. histolyticum* groups contribute less than 1 % of the total faecal microbiota of healthy adults (Franks *et al.*, 1998; Harmsen *et al.*, 1999). Our results for the *C. lituseburense* group (mean 1·5 and 2·3 %, for the baseline and 6 month samples, respectively) were thus in accordance with the results reported elsewhere. However, we found that the members of the *C. histolyticum* groups contributed a mean of 7·4 and 5·5 % (for baseline and 6 month samples, respectively) of the faecal bacterial microbiota of the 16 healthy control subjects, and 6·2 and 6·5 % (for baseline and 6 month samples, respectively) of the faecal microbiota of the IBS subjects. Satokari *et al.* (2005) note in their study that the Chis150 probe and assay conditions employed give approximately 3 % background

Table 2. Similarity values of DGGE profiles of a given subject obtained at two different time-points (baseline and 6 months later)

The values are grouped according to the RNA similarity/stability. Abbreviations: S, stable; R, rather stable; U, unstable.

Subject	Qualitative DGGE analysis						Quantitative Δclostridia (%)†, 1 versus 2
	RNA-based similarity*, 1 versus 2		DNA-based similarity*, 1 versus 2		Similarity (%)*, RNA1 versus DNA1	Similarity (%)*, RNA2 versus DNA2	
IBS subjects, diarrhoea type							
IBS 1	94·2 %	S‡§	91·9 %	R‡‖	83·2	88·6	6·7
IBS 2	38·5 %	U	44·6 %	U	88·3	59·5	50·2
IBS 8	81·0 %	R	92·0 %	R	76·8	74·1	9·0
IBS 13	50·1 %	U	87·7 %	R	67·7	70·8	5·1
IBS 14	59·9 %	U	74·4 %	U	56·0	81·9	15·5
IBS 15	89·6 %	R	90·1 %	R	77·0	89·0	15·3
IBS 16	92·5 %	S	95·1 %	S	84·4	89·2	3·3
Mean ± SD	72·3 ± 22·6		82·3 ± 17·9		76·2 ± 11·1	79·0 ± 11·4	15·0 ± 16·2
Range	38·5–94·2		44·6–95·1		56·0–88·3	59·5–89·2	3·3–50·2
IBS subjects, constipation type							
IBS 3	43·1 %	U	68·0 %	U	75·3	84·8	22·3
IBS 4	80·5 %	R	84·3 %	R	70·6	76·4	8·0
IBS 5	79·0 %	R	81·3 %	R	72·0	78·8	0·5
IBS 6	93·4 %	S	89·4 %	R	70·4	62·9	11·1
IBS 9	66·1 %	U	81·8 %	R	56·2	59·4	28·9
IBS 11¶	−¶		83·1 %	R	65·0	−¶	6·9
Mean ± SD	72·4 ± 19·0		81·3 ± 7·1		68·3 ± 6·8	72·5 ± 10·8	13·0 ± 10·6
Range	43·1–93·4		68·0–89·4		56·2–75·3	59·4–84·8	0·5–28·9
IBS subjects, mixed type							
IBS 7	94·5 %	S	85·7 %	R	70·6	69·4	8·0
IBS 10	80·6 %	R	88·0 %	R	89·5	80·7	11·7
IBS 12	83·2 %	R	87·7 %	R	66·8	63·4	20·2
Mean ± SD	86·1 ± 7·4		87·1 ± 1·3		75·6 ± 12·2	71·1 ± 8·8	13·3 ± 6·3
Range	85·7–88·0		85·7–88·0		66·8–89·5	63·4–80·7	8·0–20·2
All IBS subjects together							
Mean ± SD	75·1 ± 19·0		82·8 ± 12·3		73·1 ± 10·0	75·3 ± 10·7	13·9 ± 12·2
Range	38·5–94·5		44·6–95·1		56·0–89·5	59·4–89·2	0·5–50·2
Control subjects							
Control 1	85·9 %	R	88·8 %	R	75·1	70·7	13·0
Control 2	66·0 %	U	91·3 %	R	73·2	64·6	29·8
Control 3	87·8 %	R	90·0 %	R	85·9	84·7	42·0
Control 4	92·9 %	S	86·7 %	R	83·6	86·4	10·5
Control 5	72·2 %	R	90·1 %	R	88·7	75·3	8·6
Control 6	94·2 %	S	88·4 %	R	81·4	72·0	7·3
Control 7	91·6 %	R	58·8 %	U	42·4	68·1	18·8
Control 8	69·4 %	U	63·0 %	U	76·5	73·4	10·1
Control 9	85·5 %	R	96·3 %	S	56·9	54·2	8·8
Control 10#	−#		64·1 %	U	66·9	−#	−#
Control 11	90·7 %	R	92·2 %	S	82·3	80·8	25·1
Control 12	92·4 %	S	90·4 %	R	56·1	64·8	5·7
Control 13	92·3 %	S	84·6 %	R	88·2	86·1	1·8
Control 14	69·9 %	U	96·7 %	S	61·9	76·7	14·1
Control 15	68·2 %	U	90·2 %	R	72·8	81·5	8·7
Control 16	92·4 %	S	85·4 %	R	31·1	42·4	4·8
Mean ± SD	83·4 ± 10·8		84·8 ± 11·8		70·2 ± 16·7	72·1 ± 12·2	13·9 ± 10·8
Range	68·2–94·2		58·8–96·7		31·1–88·7	42·4–86·4	1·8–42·0

with non-target organisms when compared to the bacterial signal of the Bact338-IIA probe. Assuming that the background signal with the Chis150 probe is more or less the same from all mixed bacterial populations, the *C. histolyticum* group contributed a mean of 3–5 % of the total population, which is still higher than the previously reported <1 %. The proportions of the *C. leptum* group found in control subjects (mean 12 and 9 %, for baseline and 6 month samples, respectively) and IBS subjects (mean 12 and 15 %, for baseline and 6 month samples, respectively) were in agreement with earlier studies (Sghir *et al.*, 2000; Marteau *et al.*, 2001; Hold *et al.*, 2002).

In the present study, the *C. coccoides-E. rectale* group was the dominant subgroup of faecal clostridia. It contributed a mean of 43 % (range 18–71 %) of the total bacteria in control subjects, and 30 % (constipation type) to 50 % (diarrhoea type) (range 14–68 %) in different IBS symptom category subjects. The mean percentage observed with our control subjects is somewhat higher than the previously reported 11–35 % (range 5–45 %) for healthy adults (Franks *et al.*, 1998; Jansen *et al.*, 1999; Sghir *et al.*, 2000; Marteau *et al.*, 2001; Harmsen *et al.*, 2002a, b; Zoetendal *et al.*, 2002; Hold *et al.*, 2003; Rigottier-Gois *et al.*, 2003; Rochet *et al.*, 2004). This highlights interindividual differences that are possibly due to diet and genetic background. In addition, the age distribution in both our subject groups was wider than that of most other studies. Furthermore, it should be noted that the smaller proportions of the *C. coccoides-E. rectale* group in other studies compared to our results have been obtained with fluorescent *in situ* hybridization (FISH) [except for the studies of Sghir *et al.* (2000) and Marteau *et al.* (2001)], whereas the results of Rigottier-Gois *et al.* (2003) obtained with dot-blot hybridization (mean 22 %) were more similar to those of our study (range 12–55 % versus 18–71 % in our study). The FISH analysis measures the proportion of the cells that contain a sufficient number of ribosomes to be detected, while rRNA dot-blot hybridization gives an index that reflects the ribosomal content of the cells and the general metabolic activity (Rigottier-Gois *et al.*, 2003), as does the TRAC technique. The amount of rRNA per cell varies according to the species and the metabolic activity of the bacterial cell (Klappenbach *et al.*, 2000). When the human faecal microbiota has been analysed with the 16S rDNA library method, bacteria from the *C. coccoides-E. rectale* group have contributed 10–59 % of the total faecal flora (Wilson & Blitchington, 1996; Suau *et al.*, 1999; Hayshi *et al.*, 2002; Hold *et al.*, 2002; Wang *et al.*, 2003), which is in accordance with our results.

No difference was observed in the present study in the proportions of the *C. coccoides-E. rectale* group when all IBS subjects together were compared to the control subjects (Table 3). However, when different IBS symptom categories were compared to the control subjects, the proportion of the *C. coccoides-E. rectale* group of clostridia was significantly lower in the constipation-type IBS subjects than in the control subjects, and also at the 6 months sampling time-point of the mixed-type IBS subjects ($P < 0{\cdot}05$) (Table 3). Similar differences have been observed by Malinen *et al.* (2005). In contrast to this, Rinttilä *et al.* (2004) have found with real-time PCR that the number of cells belonging to the *C. coccoides-E. rectale* group is somewhat lower in the IBS group than in the control group. However, their subject groups contain only three subjects. As a target clostridial group, the *C. coccoides-E. rectale* group (phylogenetic clusters XIVa and XIVb) is too large to detect subtle variations between the microbiota of control and IBS subjects, and therefore needs to be divided into smaller subgroups in further studies.

Temporal stability of clostridial populations

No clear differences in intraindividual temporal stability (change in proportions between the two sampling points) of the *C. leptum*, *C. lituseburense* and *C. coccoides-E. rectale* groups were observed between the IBS and control subjects. However, the *C. histolyticum* group was temporally more stable (the change in proportions of the *C. histolyticum* groups was smaller) in mixed-type IBS subjects than in the

Table 2. cont.

*Similarity values were counted with BioNumerics 3.0 software. Clustering was performed with Pearson correlation and the unweighted pair group method with arithmetic mean (UPGMA). Amplicons with a total surface area of at least 1 % were included in the similarity analysis.
†Quantitative Δclostridia (%) indicated the total change of clostridia as measured by the TRAC technique, which was defined as: absolute change (Δ; percentage at baseline minus percentage at 6 months) in the Clept-hybridized population + absolute change (Δ) in Clit-hybridized population + absolute change (Δ) in Chis-hybridized population + absolute change (Δ) in Erec-hybridized population. See Table 1 for meanings of abbreviations.
‡Similarity value thresholds indicating stable, rather stable or unstable DGGE profiles were determined according to the visual inspection of two researchers. Similarity value thresholds for RNA-based and DNA-based profiles were determined separately.
§RNA-based similarity thresholds: stable (>92·2 %), rather stable (70·0–92·1 %), unstable (<70·0 %).
||DNA-based similarity thresholds: stable (>92·2 %), rather stable (75·0–92·1 %), unstable (<75·0 %).
¶The RNA of the second sample (time-point 6 months) of the subject IBS 11 did not amplify with RT-PCR sufficiently (for DGGE), regardless of various modifications to the protocol.
#The quality and amount of RNA of the second sample (time-point 6 months) of the subject Control 10 was not sufficient either for RT-PCR or the quantitative TRAC analysis. RNA was extracted three times with two different methods.

Table 3. Proportions of studied groups of clostridia, as quantified with the TRAC technique from faecal samples of IBS subjects and control subjects on two sampling occasions

See Table 1 for meanings of abbreviations. Values show the percentage of the hybridized total bacteria (with Bact338-IIA probe), mean ± SD. Values in bold type show a statistically significant difference ($P < 0{\cdot}05$) compared to the control subject group.

Group (number of subjects)	Probe							
	Clept1240*		Clit135†		Chis150‡		Erec482§	
	Baseline	6 months	Baseline	6 months	Baseline	6 months	Baseline	6 months
Diarrhoea type (7)								
Proportion	11·0 ± 4·7	14·0 ± 4·3	1·1 ± 2·1	0·4 ± 0·4	6·7 ± 1·5	5·3 ± 1·1	49·8 ± 10·5	45·9 ± 10·1
Range (of proportion)	4·3–16·8	8·4–22·2	0·1–5·9	0·0–1·0	5·4–9·2	4·0–7·3	38·6–68·2	30·2–60·7
Change in proportion‖		4·0 ± 6·3		1·1 ± 2·0		1·4 ± 0·8		8·5 ± 10·2
Range (of change in proportion)		0·8–17·9		0–5·6		0·1–2·7		1·6–30·9
Total change of clostridia¶								15·0 ± 16·2
Constipation type (6)								
Proportion	14·5 ± 5·4	14·8 ± 8·1	1·6 ± 1·1	3·9 ± 2·5	5·9 ± 1·6	7·1 ± 3·0	**33·8 ± 5·2**	**29·5 ± 9·4**
Range (of proportion)	5·5–20·1	5·5–25·1	0·4–3·3	0·5–8·0	3·1–7·9	3·1–10·4	24·8–38·7	14·0–40·0
Change in proportion‖		2·6 ± 2·4		2·3 ± 2·6		1·6 ± 1·5		6·4 ± 8·5
Range (of change in proportion)		0–6·1		0·1–7·4		0–3·9		0·4–23·3
Total change of clostridia¶								13·0 ± 10·6
Mixed type (3)								
Proportion	11·5 ± 4·9	17·0 ± 10·6	2·2 ± 0·6	3·2 ± 0·2	5·6 ± 1·3	6·7 ± 0·8	33·6 ± 8·4	**30·7 ± 8·1**
Range (of proportion)	7·0–16·7	8·7–29·0	1·7–2·9	3·0–3·4	4·6–7·0	5·8–7·4	27·3–43·1	22·2–38·4
Change in proportion‖		5·5 ± 5·9		1·0 ± 0·8		1·2 ± 0·8		5·7 ± 2·1
Range (of change in proportion)		1·7–12·3		0·1–1·6		0·4–1·9		4·3–8·1
Total change of clostridia¶								13·3 ± 6·3
Overall mean of IBS subjects (16)								
Proportion	12·4 ± 4·9	14·9 ± 6·8	1·5 ± 1·6	2·3 ± 2·2	6·2 ± 1·5	6·5 ± 2·1	40·7 ± 11·4	36·9 ± 12·1
Range (of proportion)	4·3–20·1	5·5–29·0	0·1–5·9	0·0–8·0	3·1–9·2	3·1–10·4	24·8–68·2	14·0–60·7
Change in proportion‖		3·8 ± 4·9		1·5 ± 2·1		1·4 ± 1·0		7·2 ± 8·2
Range (of change in proportion)		0–17·9		0–7·4		0–3·9		0·4–30·9
Total change of clostridia¶								13·9 ± 12·2
Control subjects (16)								
Proportion	11·5 ± 5·5	9·4 ± 4·2	1·7 ± 2·0	1·2 ± 1·6	7·4 ± 3·9	5·5 ± 1·8	43·3 ± 16·5	42·3 ± 13·7
Range (of proportion)	4·6–23·6	2·9–18·1	0·1–7·0	0·1–4·8	3·7–19·5	2·4–9·2	17·7–71·2	20·9–66·9
Change in proportion‖		3·3 ± 4·1		1·0 ± 1·6		2·7 ± 2·9		9·2 ± 8·3
Range (of change in proportion)		0–13·2		0–6·4		0·4–12·6		0·2–24·6
Total change of clostridia¶								13·9 ± 10·8

*The Clept1240 probe (Sghir *et al.*, 2000) targets the *C. leptum* group, which corresponds to clostridial phylogenetic cluster IV (Collins *et al.*, 1994).

†The Clit135 probe (Franks *et al.*, 1998) targets the *C. lituseburense* group, which corresponds to clostridial phylogenetic cluster XI (Collins *et al.*, 1994).

‡The Chis150 probe (Franks *et al.*, 1998) targets the *C. histolyticum* group, which corresponds to clostridial phylogenetic clusters I and II (Collins *et al.*, 1994).

§The Erec482 probe (Franks *et al.*, 1998) targets the *C. coccoides-E. rectale* group, which corresponds to clostridial phylogenetic clusters XIVa and XIVb (Collins *et al.*, 1994).

‖The change was defined as: absolute change (Δ; percentage at baseline minus percentage at 6 months) in the population.

¶The total change of clostridia was defined as: absolute change (Δ; percentage at baseline minus percentage at 6 months) in Clept-hybridized population + absolute change (Δ) in Clit-hybridized population + absolute change (Δ) in Chis-hybridized population + absolute change (Δ) in Erec-hybridized population.

control subjects ($P = 0{\cdot}05$) (Table 3). Altogether, there was mostly smaller temporal variation in the studied clostridial groups than interindividual variation, and this has also been reported by Matsuki *et al.* (2004). However, the instability of predominant bacteria was also seen in the change of the proportions of the studied clostridial groups (Tables 2 and

3) in our study. Subjects whose predominant bacterial population (by RT-PCR-DGGE) was unstable over the studied time-period had a significant change in the proportions of the clostridial groups between the two sampling points (baseline and 6 months) when compared to the subjects whose predominant bacterial population was stable ($P < 0{\cdot}05$). This might be expected, since the studied clostridial groups represented the dominant faecal microbiota in these subjects. The same was also noticed with DNA-based DGGE profiles, but the difference with DNA-based profiles was not statistically significant ($P = 0{\cdot}06$).

Conclusions

The differences in faecal bacterial population between IBS and control subjects have been reported in several studies (Balsari *et al.*, 1982; Bradley *et al.*, 1987; Madden & Hunter, 2002; Mättö *et al.*, 2005). We found a marked difference in the proportions of the *C. coccoides-E. rectale* group of clostridia between constipation-type IBS subjects and control subjects. In addition, there was more instability in the DGGE profiles derived from faecal RNA amplicons of the IBS subjects than those of the control subjects. However, further studies are needed with larger subject groups to confirm these findings.

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ARTICLE II

PCR-DGGE and RT-PCR-DGGE show diversity and short-term temporal stability in the *Clostridium coccoides* ***Eubacterium rectale*** **group in the human intestinal microbiota**

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PCR DGGE and RT-PCR DGGE show diversity and short-term temporal stability in the *Clostridium coccoides–Eubacterium rectale* group in the human intestinal microbiota

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Abstract

As the *Clostridium coccoides–Eubacterium rectale* (Erec; clostridial phylogenetic cluster XIVa) group is one of the major groups of the human intestinal microbiota, DNA- and RNA-based population analysis techniques (denaturing gradient gel electrophoresis; DGGE) were developed and applied to assess the diversity and temporal stability (6 months–2 years) of this faecal clostridial microbiota in 12 healthy adults. The stability of the Erec group was compared with the stability of the predominant bacterial microbiota, which was also assessed with PCR-DGGE. In addition, the Erec group was quantified with a hybridization-based method. According to our results, the Erec group was diverse in each subject, but interindividual uniqueness was not as clear as that of the predominant bacteria. The Erec group was found to be temporally as stable as the predominant bacteria. Over 200 clones obtained from two samples proved the developed method to be specific. However, the amount of bacteria belonging to the Erec group was not related to the diversity of that same bacterial group. In conclusion, the newly developed DGGE method proved to be a valuable and specific tool for the direct assessment of the stability of the Erec group, demonstrating diversity in addition to short-term stability in most of the subjects studied.

Introduction

Clostridia are phylogenetically and metabolically highly diverse bacteria, which together with their close relatives constitute a predominant fraction of the faecal microbiota of human adults (Wilson & Blitchington, 1996; Franks *et al*., 1998; Zoetendal *et al*., 1998; Suau *et al*., 1999). The genus *Clostridium* currently contains 146 species. Clostridia and phylogenetically related bacteria are divided into 19 distinct clusters (Collins *et al*., 1994), and most of the clusters contain species from genera other than *Clostridium*, for example *Eubacterium*, *Ruminococcus*, *Coprococcus*, *Peptostreptococcus*, and *Faecalibacterium*, which are also commonly found in the colonic microbiota (e.g. Suau *et al*., 1999). The 16S rRNA gene-based phylogeny of clostridia is still incomplete, and several prevalent species within these genera have been reclassified as they have been studied more closely (e.g. Wade *et al*., 1999). Studies on the characterization of faecal microbiota of healthy humans by 16S rRNA gene sequencing have revealed abundantly, clones of uncultured bacteria that are distributed in the clostridial clusters (Suau *et al*., 1999; Eckburg *et al*., 2005). The predominant intestinal clostridia belong mainly to clusters XIVa [*Clostridium coccoides–Eubacterium rectale* (Erec) group] and IV (*Clostridium leptum* group) (Franks *et al*., 1998; Suau *et al*., 1999; Sghir *et al*., 2000; Eckburg *et al*., 2005), whereas most of the medically important clostridia belong to cluster I (Stackebrandt *et al*., 1999). Many clostridia are cultivable, but their culture-based quantification is impractical owing to the lack of good subgroup-selective media (with the exception of some clinically important species). Hence, culture-independent techniques have great practical relevance in the study of clostridia (Satokari *et al*., 2005).

Clostridia contain saccharolytic and/or proteolytic species and are able to metabolize a wide variety of substrates (Cato *et al*., 1986). In the colon they are likely to have an important role in the fermentation and putrefaction of food-derived substances, resulting in various metabolites such as fatty acids and gases (Konstantinov *et al*., 2005). Short-chain fatty acids formed by microbial fermentation have an important effect on colonic health. Butyrate, in

particular, has an important role in the metabolism and normal development of colonic epithelial cells and it has been implicated in protection against cancer and ulcerative colitis (Cummings & MacFarlane, 1997). Barcenilla *et al.* (2000) showed that 80% of the butyrate-producing isolates of human faecal origin belonged to the clostridial phylogenetic cluster XIVa. On the other hand, *Clostridium bolteae*, which also belongs to the clostridial cluster XIVa, is postulated to have a role in childhood autism (Song *et al.*, 2004).

The aim of the present study was to develop both DNA- and RNA-based denaturing gradient gel electrophoresis (DGGE) methods using 16S rRNA and its corresponding gene for the detection of the diversity and temporal stability of the Erec group (clostridial phylogenetic cluster XIVa) from human faeces. In addition, the proportion of the Erec group in the total microbiota was evaluated with a recently developed multiplexed and quantitative hybridization-based technique (Satokari *et al.*, 2005). Furthermore, the diversity and temporal stability of the predominant bacterial microbiota were studied with both DNA- and RNA-based DGGE to assess the possible correlation between the temporal stability of the *E. rectale–C. coccoides* group and the predominant microbiota.

Materials and methods

Bacterial strains

The bacterial reference strains used for the optimization and validation of PCR and PCR-DGGE of the Erec group (phylogenetic cluster XIVa; Collins *et al.*, 1994) are described in Table 1. The reference strains were grown on media recommended by the culture collections providing the strains.

Human faecal samples

The subject group consisted of three male and nine female subjects who were 34–63 years of age. Faecal samples were obtained on two occasions 6 months apart (0 and 6 months; subjects 5–12). For a longer-term stability study, samples were obtained on four occasions (0, 3, 6, and 24 months; subjects 1–4). The main recruiting criterion was a good (normal) intestinal balance [absence of repeating and/or persisting gastrointestinal (GI) symptoms]. The exclusion criteria were regular GI-tract symptoms, lactose intolerance, celiac disease, and antimicrobial therapy during the 2 months immediately prior to each sampling point. The subjects defaecated into a plastic container, which was then 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 and processed in an anaerobic workstation (Don Whitley Scientific Ltd, Shipley, UK) within 0–4 h of the defaecation. The samples were maintained at $-70\,^{\circ}C$ until analysed.

The study was approved by the ethical committee of VTT Technical Research Centre of Finland, Espoo, Finland.

DNA extraction

DNA was extracted from reference strains and 300 mg of faecal material using a FastDNA Spin Kit for Soil (QBIOgene, Carlsbad, CA) according to the manufacturer's instructions but with one modification: the bacterial cells were broken with a Fast Prep instrument (Bio 101 Savant, Holbrook, NY) at $6.0\,m\,s^{-1}$ for 60 s from one (reference strains) to three (some clostridial reference strains and human faecal samples) times. The isolated DNA was stored at $-20\,^{\circ}C$ until examined.

RNA extraction and biotinylation

RNA was extracted from reference strains and faeces as previously described by Satokari *et al.* (2005) and further purified using the clean-up protocol of the RNeasy mini kit (Qiagen, Hilden, Germany). RNA concentration and purity were determined spectrophotometrically ($A_{260\,nm}/A_{280\,nm}$; BioPhotometer 6131, Eppendorf, Hamburg, Germany), and the integrity of RNA was evaluated with agarose gel electrophoresis in which 23S and 16S RNA were observed.

Purified RNA used for quantitative analysis (TRAC; see below) was biotinylated using Photoprobe® Biotin (Vector Laboratories, CA). Biotinylation was performed by exposing the reaction to long-wave UV light (365 nm) for 30 min, and the subsequent purification of the RNA from free biotin was performed according to the manufacturer's instructions and as described by Satokari *et al.* (2005).

PCR and reverse transcription PCR of the Erec group

Twenty-four primer combinations, four $MgCl_2$ concentrations, BSA and formamide additions, 11 annealing temperatures, and three cycle numbers were tested in preliminary experiments. The partial 16S rRNA gene of the *C. coccoides–E.rectale* group was subsequently PCR-amplified for DGGE using primer pairs Ccoc-f and Ccoc-r+GC in addition to Ccoc-f+GC and Ccoc-r (Table 2). Optimized PCR amplifications were performed in a total volume of 50 μL containing 1 μL of appropriately diluted template DNA, 0.4 μM of both primers, 0.2 mM dNTP, and 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 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 54 °C for 30 s, and elongation at 72 °C for 60 s, and a final extension at 72 °C for 30 min. PCR products of the expected size were obtained with both primer pairs. Primers Ccoc-f and Ccoc-r+GC

Table 1. Bacteria used in the validation of *Clostridium coccoides–Eubacterium rectale*-specific PCR and the results of the PCR specificity tests

Clostridial cluster*	Species	Strain†	PCR result‡
XIV	*Anaerostipes caccae*	VTT E-052773 (DSM 14662)	+
XIV	*Clostridium bolteae*	VTT E-052776 (DSM 15670)	+
XIV	*Clostridium clostridioforme*	VTT E-052777 (DSM 933)	+
XIV	*Clostridium coccoides*	VTT E-052778 (DSM 935)	+
XIV	*Clostridium indolis*	VTT E-042445 (DSM 755)	+
XIV	*Clostridium symbiosum*	VTT E-981051 (DSM 934)	+
XIV	*Dorea longicatena*	VTT E-052788 (DSM 13814)	+
XIV	*Eubacterium eligens*	VTT E-052844 (DSM 33769)	+§
XIV	*Eubacterium hallii*	VTT E-052783 (DSM 3353)	+
XIV	*Eubacterium ramulus*	VTT E-052783 (DSM 15684)	+
XIV	*Lachnospira multipara*	VTT E-052784 (DSM 3073)	+
XIV	*Roseburia intestinalis*	VTT E-052785 (DSM 14610)	+
XIV	*Ruminococcus productus*	VTT E-052786 (DSM 2950)	+¶
I	*Clostridium acetobutylicum*	VTT E-93498	–
I	*Clostridium butyricum*	VTT E-97426 (DSM 10702)	–
I	*Clostridium perfringens*	VTT E-98861 (ATCC 13124)	–
II	*Clostridium histolyticum*	VTT E-052779 (DSM 2158)	–
IV	*Clostridium leptum*	VTT E-021850 (DSM 753)	–
IX	*Megasphera elsdenii*	VTT E-84221 (DSM 20460)	–
IX	*Pectinatus cerevisiiphilis*	VTT E-79103 (ATCC 29359)	–
IX	*Selenomonas lacticifex*	VTT E-90407 (DSM 20757)	–
XI	*Clostridium lituseburense*	VTT E-021853 (DSM 797)	–
XIX	*Fusobacterium necrophorum*	VTT E-001739 (ATCC 25286)	–
XIX	*Fusobacterium nucleatum* ssp. *nucleatum*	VTT E-052770 (RHI 4184)	–
	Atopobium parvulum	VTT E-052774 (DSM 20469)	–
	Bacteroides fragilis	VTT E-022248 (DSM 2151)	–
	Bifidobacterium longum	VTT E-96664 (DSM 20219)	–
	Collinsella aerofaciens	VTT E-052787 (DSM 3979)	–
	Desulfovibrio desulfuricans ssp. *desulfuricans*	VTT E-95573 (DSM 642)	–
	Eggerthella lenta	VTT E-001735 (ATCC 25559)	–
	Enterococcus faecalis	VTT E-93203 (DSM 20478)	–
	Enterococcus faecium	VTT E-93204 (DSM 20477)	–
	Escherichia coli	VTT E-94564 (DSM 30083)	–
	Lactobacillus salivarius	VTT E-97853 (DSM 20555)	–
	Klebsiella terrigena	VTT E-96696	–
	Pediococcus acidilactici	VTT E-93493 (DSM 20284)	–
	Prevotella melaninogenica	VTT E-052771 (ATCC 25845)	–
	Veillonella parvula	VTT E-001737 (ATCC 10790)	–

*Clostridial phylogenetic cluster number (Collins *et al.*, 1994).
†DSM, www.dsmz.de; ATCC, www.lgcpromochem-atcc.com; VTT, www.inf.vtt.fi/pdf/tiedotteet/1999/T1980.pdf.
‡+, positive PCR result; –, negative PCR result. Each positive strain produced one strong band in DGGE unless otherwise stated.
§Three strong bands in the DGGE profile, one band in agarose gel after PCR.
¶Two strong bands in the DGGE profile, one band in agarose gel after PCR.

were used for the reverse transcription PCR (RT-PCR) (Table 2). RT-PCR was performed with the Qiagen® One-Step RT-PCR Kit (Qiagen) according to the manufacturer's instructions. The PCR program consisted of reverse transcription performed at 50 °C for 30 min, followed by denaturation at 95 °C for 15 min. Thereafter the RT-PCR program was similar to the PCR-program: 30 cycles of denaturing at 94 °C for 45 s, primer annealing at 54 °C for 30 s and elongation at 72 °C for 60 s, and a final extension at 72 °C for 30 min.

PCR and RT-PCR of the predominant microbiota

The partial 16S rRNA gene was PCR-amplified using primers U968-f+GC and U1401-r (Table 2) as described by Mättö *et al.* (2005). RT-PCR was performed with the Qiagen® OneStep RT-PCR Kit according to the manufacturer's instructions. Primers used for the RT-PCR were the same as those used for the PCR (Table 2). The PCR program consisted of reverse transcription performed at 50 °C for 30 min, followed by denaturation at 95 °C for 15 min.

Table 2. Probes and primers used in the present study

Target group	Short name	Probe/primer	Use	Sequence (5′ → 3′)	Reference or source
Clostridium coccoides–Eubacterium rectale group*	Erec group	Erec482-5A†	TRAC	GCTTCTTAGTCARGTACCG AAAAA	Franks *et al*. (1998) and Satokari *et al*. (2005)
Clostridium coccoides–Eubacterium rectale group‡	Erec group	Ccoc-f	PCR-DGGE	AAATGACGGTACCTGACTAA	Matsuki *et al*. (2002)
Clostridium coccoides–Eubacterium rectale group‡	Erec group	Ccoc-f+GC	PCR-DGGE	CGCCCGGGGCGCGCCCCG GGCGGGGCGGGGGCA CGGGGGGAAATGACGG TACCTGACTAA	This study
Clostridium coccoides–Eubacterium rectale group‡	Erec group	Ccoc-r	PCR-DGGE	CTTTGAGTTTCATTCTTGCGAA	Matsuki *et al*. (2002)
Clostridium coccoides–Eubacterium rectale group‡	Erec group	Ccoc-r+GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGG CGGGGCGGGGGCACGGGGGG CTTTGAGTTTCATTCTTGCGAA	This study
Bacteria		Bact338-IIA§	TRAC	GCTGCCTCCCGTAGGAGTIIA	Amann *et al*. (1990) and Satokari *et al*. (2005)
Bacteria¶		U968-f+GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGG CGGGGCGGGGGCACGGGGGG AACGCGAAGAACCTTA	Nübel *et al*. (1996)
Bacteria¶		U1401-r	PCR-DGGE	CGGTGTGTACAAGACCC	Nübel *et al*. (1996)
Sequencing		T7	Seq.	TAATACGACTCACTATAGG	Promega
Sequencing		SP6	Seq.	GATTTAGGTGACACTATAG	Promega

*Clostridial phylogenetic clusters XIVa and XIVb (Collins *et al.*, 1994).
†The Erec482 (Franks *et al.*, 1998) probe was tailed with an additional AAAAA sequence (Erec482-5A) in the 3′-end for size distinction purposes.
‡Clostridial phylogenetic cluster XIVa (Collins *et al.*, 1994).
§The Bact338 (Amann *et al.*, 1990) probe was tailed with an additional IIA sequence (Bact338-IIA, where I is inosine) in the 3′-end for size distinction purposes.
¶Partial 16S rRNA gene (V_6–V_8 hypervariable region).

Thereafter the RT-PCR program was similar to the PCR program: 35 cycles of denaturing at 94 °C for 30 s, primer annealing at 50 °C for 20 s and elongation at 72 °C for 40 s, and a final extension at 72 °C for 10 min.

Specific quantification of 16S rRNA gene

Multiplexed quantification of clostridial 16S rRNA gene was performed with the TRAC technique (transcript analysis with the aid of affinity capture) according to Satokari *et al*. (2005). Specific 16S rRNA gene-targetted probes for different groups of clostridia (phylogenetic clusters I and II, IV, XI and XIVa and b; Collins *et al*., 1994) and a universal bacterial probe were used in multiplexed hybridization (Table 2; however, only phylogenetic cluster XIVa and b is discussed in this study).

Cloning of the PCR-amplified products

PCR amplicons generated with the set of Erec-group primers were purified using a Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Two samples were selected based on the TRAC results – one sample containing the highest percentage of Erec-group bacteria and another with a low percentage. DNA purity and yield were estimated by electrophoresis in 1% (w/v) agarose gels. Purified amplification products were ligated into pGEM-T vector system II and transformed into *Escherichia coli* JM109 high-efficiency competent cells (Promega, Madison, WI) according to the manufacturer's instructions. One hundred and thirty randomly picked colonies of ampicillin-resistant transformants from each sample were further subcultured on Luria–Bertani (LB) agar (Atlas, 1997) plates supplemented with ampicillin (100 μg mL^{-1}; Sigma, St Louis, MO), X-gal (100 μg mL^{-1}; Promega), and IPTG (0.5 mM; Promega) and incubated at 37 °C overnight. A loop full of bacterial mass was transferred into 50 μL of Tris-EDTA and incubated at 95 °C for 15 min to lyse the cells. PCR with pGEM-T-specific primers T7 and SP6 (Table 2) was performed from the lyzed cells to check the size of the inserts. The PCR products of the plasmids containing inserts of the right size were purified with the Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions and sequenced with an ABI PRISM BigDye terminator cycle sequencing kit v.3.1 (Applied Biosystems,

Foster City, CA) according to the manufacturer's instructions using the primer T7. All the sequences were analysed with an ABI PRISM 3100 automated capillary DNA cycle sequencer (Applied Biosystems) and checked and edited with the CHROMAS program (Technelysium Pty Ltd, Helensvale, Australia). CLUSTALW (http://align.genome.jp) was used for the alignment of the sequences and for checking of the sequence similarities. All unequal sequences were thereafter identified through the GenBank database (www.ncbi.nlm.nih.gov) using the BLAST (basic local alignment search tool) algorithm (Altschul *et al*., 1997) and using the 'Classifier' tool of the Ribosomal Database Project (RDP) II (Cole *et al*., 2005). CLUSTALW and DNAMAN 4.1 (Lynnon BioSoft) were used for the creation of operational taxonomic units (OTUs). An OTU, as employed here, consisted of all sequences with less than 2% divergence from 440 aligned homologous sequences (Suau *et al*., 1999). Phylogenetic analysis of OTU subgroups was performed with DNAMAN 4.1. One representative of each OTU was deposited in the GenBank database, and the sequences are available under the accession numbers DQ307759–DQ307802.

DGGE analysis of 16S rRNA gene fragments

DGGE analysis of predominant bacteria was performed as described by Mättö *et al*. (2005). The primer pair Ccoc-f and Ccoc-r+GC was chosen for the analysis of the samples with DGGE because of specificity and optimal migration. DGGE analysis of the Erec group of clostridia was similar to the DGGE analysis of predominant bacteria. Various denaturing gradients were tested and subsequently a denaturing gradient from 38 to 60% [where 100% is 7 M urea and 40% (v/v) deionized formamide] was chosen because of optimal migration and differentiation. The similarity of the PCR-DGGE profiles of the samples obtained from a single subject at different sampling points was compared in order to evaluate the temporal stability of the predominant and clostridial faecal bacterial populations. The comparison of the profiles was performed by calculating a similarity percentage using BIONUMERICS software version 4.01 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Clustering was performed with Pearson correlation and the unweighted-pair group method (UPGMA). Amplicons with a total surface area of at least 1% were included in the similarity analysis.

After sequence analysis of the clones, all the clones with different sequences were subjected to Erec-PCR-DGGE, after which the migration of each clone was compared with the migration of different amplicons in the original sample.

Statistical analysis

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

Results

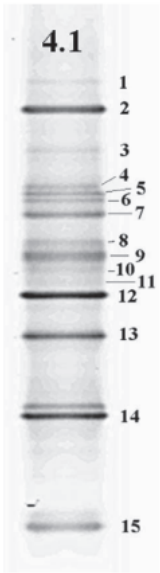
Optimization and validation of PCR-DGGE and RT-PCR-DGGE of the Erec group

When the specificity of primer pairs Ccoc-f – Ccoc-r+GC and Ccoc-f+GC – Ccoc-r (Table 2) was evaluated with reference strains, the primers gave positive PCR results for all the target bacteria belonging to the clostridial phylogenetic cluster XIVa (Table 1). No false-positive results were obtained (Table 1). In most cases only one strong or one strong and one additive weak band was/were seen in the DGGE profiles of the reference strains. However, the DGGE profiles of *Ruminococcus productus* and *Eubacterium eligens* contained two and three strong bands, respectively (Table 1). Most of the amplicons of the used reference strains migrated differently and could thus be distinguished. However, the amplicons of *Clostridium bolteae*, *Clostridium clostridioforme*, and *C. coccoides* migrated to the same position (data not shown).

Two samples were cloned after Erec-PCR to validate the specificity of the newly developed method. All the sequenced 205 clones belonged to the Erec clostridial cluster XIVa, as expected. The 108 clones of subject 4 fell into 21 OTUs, and the 97 clones of subject 6 into 22 OTUs, when an OTU was defined as a phylogenetic group that consisted of members having over 98% sequence similarity to each other. The DGGE profiles of the cloned samples and the sequence information obtained from the cloning and Erec-DGGE are presented in Figs 1 and 2. OTU 5 (band number 9) of subject 4 was divided into two subgroups: OTUs 5A and 5B, since, according to the BLAST results, OTUs 5A and 5B had 100% similarity to different species (although having over 98% similarity to each other; Fig. 1). Similarly, OTU 21 (band numbers 3, 7, 11, 12, and 13) of subject 6 was divided into subgroups according to phylogenetic analysis and migration in the DGGE gel (Fig. 2). When cloned samples 4.1 and 6.1 were run in the same DGGE gel, several amplicons migrated similarly – for example band number 2 of sample 4.1 migrated similarly to band number 2 of sample 6.1, and both were identified as *Anaerostipes* IE4 (Fig. 1).

Proportion, diversity and temporal stability of the *C. coccoides–E. rectale* group from faecal samples of healthy adults

DNA-based PCR-DGGE and RNA-based RT-PCR-DGGE analyses targetted to the *C. coccoides–E. rectale* group showed intraindividual diversity of the faecal microbiota (Fig. 3). In addition, the interindividual profiles were divergent (Figs 3 and 4). After comparison of both DNA-



Band number	OTU	Blast-result	similarity	clones /OTU	Band number in sample 6.1
1 & 2 [a]	1	*Anaerostipes* IE4	97-98 %	13	2
3, 4 [b]	8	*Ruminococcus lactaris*	96 %	5	
5	21	*Clostridium hathawayi*	97 %	1	
6	11	*Clostridium nexile*	99 %	5	5
7	2	*Eubacterium hallii*	98 %	5	
7	7	*Eubacterium oxidoreducens*	96 %	6	
8	3	*Ruminococcus* sp. CE2	96 %	2	7
		Ruminococcus schinkii	94 %		
9	5A	*Roseburia faecalis*	99-100 %	8	8
	5B	*Roseburia intestinalis*	100 %	2	
9	6	*Eubacterium rectale*	100 %	1	8
10	10	*Dorea formigerans*	99 %	1	
11	14	*Clostridium* sp. cTPY-17	97 %	8	
		Ruminococcus obeum / schinkii / torques / lactaris	94-96 %		
12	4	*Ruminococcus* sp. CE2	98-100 %	12	13
		R. obeum / R. schinkii	94-96 %		
13	18	*Clostridium clostridioforme*	98 %	7	14
14 [c]	9	*Ruminococcus lactaris*	99 %	1	15
14 [c]	16	*Ruminococcus obeum*	97 %	13	
14 [c]	17	*Ruminococcus productus*	95 %	4	
15	13	*Ruminococcus gnavus*	96 %	4	
n.d.	12	*Ruminococcus gnavus*	99 %	1	
n.d.	15	*Ruminococcus obeum*	99 %	1	
n.d.	19	*Clostridium amygdalinum / indolis*	96 %	1	
n.d.	20	*Clostridium hathawayi*	95 %	1	

[a] 12 clones migrated as band 2 and one clone as band 1
[b] 3 clones migrated as band 3, 1 clone as band 4 and 1 clone a little above band 6
[c] a double band

Fig. 1. The cloned and sequenced bands from the *Clostridium coccoides–Eubacterium rectale*-specific PCR-DGGE of the baseline sample of subject 4 (n.d., the clone is not visible in the community profile).

and RNA-based profiles with BioNumerics software – using Pearson correlation for similarity analysis and UPGMA for clustering of the profiles – all samples of a given subject clustered together in most of the cases (nine out of 12 subjects). Only samples of three subjects (subjects 1, 9, and 11) clustered within another subject's cluster (Fig. 4). The DNA-based profiles of 10 out of 12 subjects and the RNA-based profiles of eight out of 12 subjects were stable or rather stable in the 6-month follow-up study (Fig. 4). When DNA- and RNA-based profiles from the same time point of a given subject were compared, nine DNA vs. RNA profiles out of 12 from baseline and 10 out of 12 from the 6-month sampling point were similar (data not shown). The long-term study (2-year period) showed that the DNA-based profiles of subjects 3 and 1 were unstable or rather unstable, respectively, and the profiles of subjects 2 and 4 contained minor differences (Figs 3 and 5).

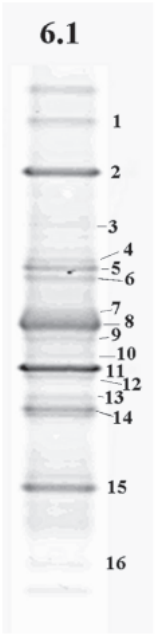
The *C. coccoides–E. rectale* group comprised an average of 45% (range 18–71%) of the total bacteria from human faecal samples as detected with the hybridization-based TRAC technique (Table 3). There were clear differences in intraindividual temporal stability (change in proportions between the two sampling points; Table 3).

Stability and diversity of the predominant bacteria from faecal samples of healthy adults

Both DNA- and RNA-based DGGE analyses targetted to the predominant bacterial population showed considerable intraindividual diversity as well as uniqueness of the faecal microbiota. The DNA- and RNA-based profiles in the follow-up study (6 months) were stable or rather stable in nine out of 12 subjects (data not shown), and in the DNA-based long-term study (2 years) in two out of four subjects (Fig. 5). When DNA- and RNA-based profiles from the same time point of a given subject were compared, the temporal intraindividual similarity values of RT-PCR-DGGE and PCR-DGGE profiles were significantly higher than the intraindividual similarity values between DNA- and RNA-based DGGE profiles ($P < 0.05$) (data not shown). However, since each individual had unique PCR-DGGE and RT-PCR-DGGE profiles, the intraindividual RNA- and DNA-based DGGE profiles resembled each other more than the interindividual RNA-based profiles (data not shown).

Discussion

The aim of this study was to develop both DNA- and RNA-based population analysis techniques (DGGE) for the



Band number	OTU	Blast-result	similarity	clones /OTU
1	11	*Ruminococcus torques*	98 %	1
2	7	*Anaerostipes* sp. IE4	98 %	4
3	21A	*Ruminococcus* sp. CE2	97 %	1[a]
		Eubacterium rectale / Roseburia faecalis	95 %	
4	10	*Ruminococcus lactaris*	96 %	4
5	13	*Clostridium nexile*	99 %	9
6	6	*Eubacterium hallii*	98 %	4
7	21B	*Ruminococcus* sp. CE2	98 %	1[a]
		Ruminococcus obeum / schinkii	96&	
8	16	*Eubacterium rectale*	99-100%	15
8	18	*Roseburia faecalis*	99-100%	10
9	14	*Ruminococcus obeum / schinkii*	96 %	4
10	22	*Eubacterium rectale*	95 %	1
11	21C	*Ruminococcus* sp. CE2	98 %	9[a]
		Ruminococcus obeum / schinkii	96 %	
12	21D	*Ruminococcus* sp. CE2	99 %	4[a]
		Ruminococcus obeum / schinkii	97 %	
13	21E	*Ruminococcus* sp. CE2	99 %	3[a]
		Ruminococcus obeum / schinkii	96 %	
14	1	*Clostridium clostridioforme*	98 %	7
15	5	*Ruminococcus productus / schinkii*	95 %	4
15[b] (& 9)	4	*Ruminococcus obeum*	96 %	1
15[b]	2	*Clostridium amygdalinum / Cl. indolis / Ruminococcus obeum*	96 %	1
16	12	*Ruminococcus gnavus*	97 %	1
n.d.	3	*Ruminococcus obeum*	96 %	1
n.d.	8	*Clostridium hathawayi / Hespellia porcina*	95 %	1
n.d.	9	*Clostridium amygdalinum / jejuense / algidixylanolyticum*	95 %	1
n.d.	15	*Ruminococcus obeum*	96 %	1
n.d.	17	*Eubacterium rectale*	97 %	1
n.d.	19	*Roseburia faecalis*	96-97 %	2
n.d.	20	*Eubacterium rectale*	97 %	1
n.d.	21F	*Ruminococcus sp. CE2*	98 %	3[a]
		Ruminococcus obeum / schinkii	96 %	

[a] There were altogether 21 clones in OTU 21
[b] a double band

Fig. 2. The cloned and sequenced bands from the *Clostridium coccoides–Eubacterium rectale* group-specific PCR-DGGE of the baseline sample of subject 6 (n.d., the clone is not visible in the community profile).

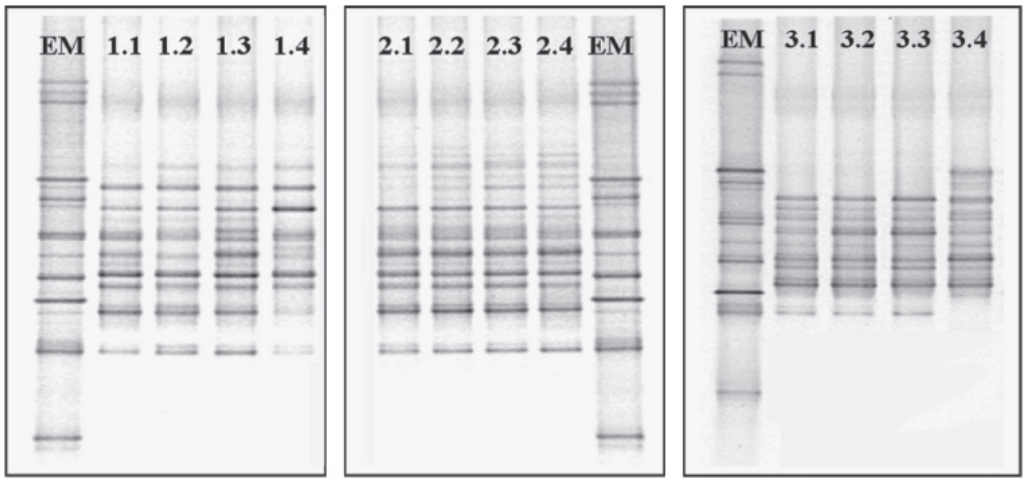


Fig. 3. DNA-based long-term stability study from faeces of three healthy adults with *Clostridium coccoides–Eubacterium rectale* group-specific PCR-DGGE. The samples were obtained at time points 0 (1.1, 2.1, 3.1), 3 months (1.2, 2.2, 3.2), 6 months (1.3, 2.3, 3.3), and 2 years (1.4, 2.4, 3.4). EM, Erec marker.

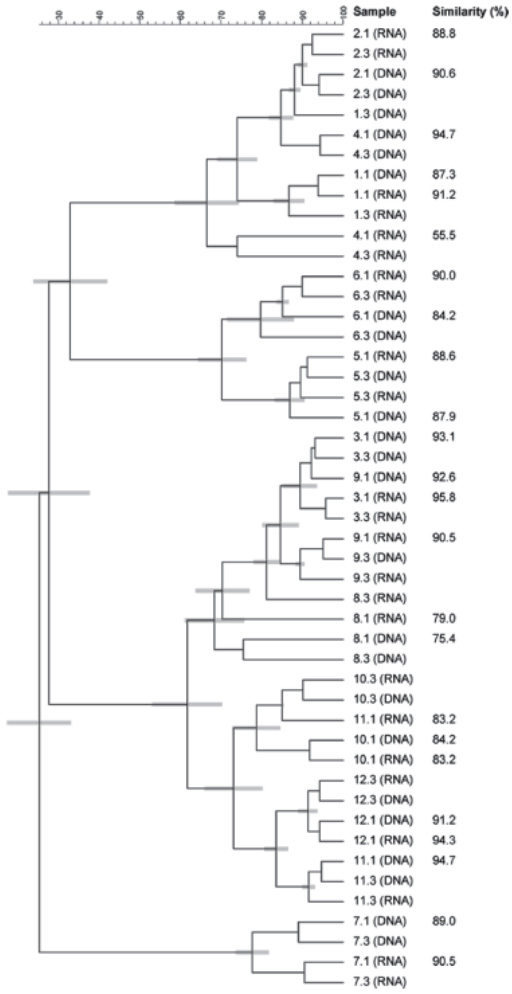


Fig. 4. Similarities of the DNA- and RNA-based DGGE profiles of the *Clostridium coccoides–Eubacterium rectale* group of human faeces. The samples were taken on two occasions: baseline (RNA1 and DNA1) and 6 months later (RNA3 and DNA3). The comparison of the profiles was performed by calculating the similarity percentage using BioNumerics software version 4.01. The similarity percentage presented indicates either DNA- or RNA-based similarity between the DGGE profiles of the two sampling points (baseline and 6 months) of the same individual. DNA-based similarity values are represented in the same row as the DNA-based baseline sample, and RNA-based similarity values in the same row as the RNA-based baseline sample. Clustering was performed with Pearson correlation and the UPGMA method. Amplicons with a total surface area of at least 1% were included in the similarity analysis. The grey bars represent the error bars.

detection of diversity and temporal stability of the Erec group (Erec group; clostridial phylogenetic cluster XIVa; Collins *et al.*, 1994) from human intestinal samples. To our knowledge, this is the first study to use DGGE for studying the diversity of the Erec group. The Erec group is one of the major bacterial groups in human faeces (e.g. Wilson & Blitchington, 1996; Franks *et al.*, 1998; Suau *et al.*, 1999; Vaughan *et al.*, 2000), and before this study it was only possible to quantify the number of bacteria belonging to this group with either hybridization-based methods (Franks *et al.*, 1998; Seksik *et al.*, 2003; Satokari *et al.*, 2005) or real-time PCR (Matsuki *et al.*, 2004; Rinttilä *et al.*, 2004; Song *et al.*, 2004). DGGE methods have already been developed for other relevant faecal/GI bacterial groups/species – namely bifidobacteria (Satokari *et al.*, 2001), the *Lactobacillus* group (Walter *et al.*, 2000; Heilig *et al.*, 2002; Vanhoutte *et al.*, 2004), the *Bacteroides fragilis* group (Vanhoutte *et al.*, 2004), and predominant bacteria (e.g. Nübel *et al.*, 1996). Because there is extensive evidence that Erec-group bacteria are abundant in the human intestinal microbiota, we developed a simple method (PCR-DGGE) to assess the diversity of this group, which does not necessitate the building of a clone library from each sample. The new DGGE method was validated with a clone library, which consisted of 205 clones originating from two subjects (one sample per subject). All 205 sequenced clones proved to belong to the clostridial phylogenetic cluster XIVa. All the amplicons seen in the profiles were identified through the clone library, and each DGGE band consisted of either one OTU or a few OTUs that were phylogenetically closely related (although several bacterial species migrated similarly). Therefore the number of amplicons seen in the DGGE profiles correctly reflected the clostridial phylogenetic diversity of the samples. The majority of the sequences retrieved from the 205 clones of the two subjects were different. This has also been shown to be the case in other studies in which extensive numbers of predominant bacterial clones from different individuals were sequenced (Eckburg *et al.*, 2005).

We also studied the number of bacteria belonging to the Erec group with a hybridization-based method (TRAC; Satokari *et al.*, 2005). The average percentage of the Erec group observed in healthy adults ($45 \pm 10\%$) in this study was somewhat higher than what has previously been reported with FISH, namely 11–35% (Franks *et al.*, 1998; Jansen *et al.*, 1999; Sghir *et al.*, 2000; Marteau *et al.*, 2001; Harmsen *et al.*, 2002a, b; Zoetendal *et al.*, 2002; Hold *et al.*, 2003; Rigottier-Gois *et al.*, 2003; Rochet *et al.*, 2004). The proportion of the Erec-group bacteria varied greatly within our study group (18–71%), and it has been reported that, when the human faecal microbiota has been analysed with the 16S rRNA gene library method, bacteria from the Erec group have comprised 10–59% of the total faecal flora (Wilson & Blitchington, 1996; Suau *et al.*, 1999; Hayshi *et al.*, 2002; Hold *et al.*, 2002; Wang *et al.*, 2003). These results are more in accordance with our results. The two samples that were cloned in this study were chosen based on

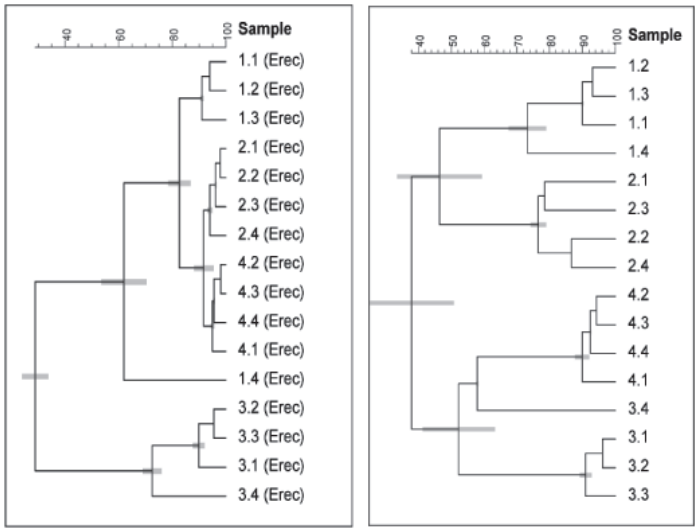


Fig. 5. Similarities of the DNA-based DGGE profiles of the long-term (24 months) study. The dendogram on the left is based on the profile similarities of the *Clostridium coccoides–Eubacterium rectale* group, whereas the dendogram on the right is based on the profile similarities of the predominant bacterial microbiota. The comparison of the profiles was performed by calculating the similarity percentage using BioNumerics software version 4.01. Clustering was performed with Pearson correlation and the UPGMA method. Amplicons with a total surface area of at least 1% were included in the similarity analysis. The samples were obtained at time points 0 (1.1, 2.1, 3.1, 4.1), 3 months (1.2, 2.2, 3.2, 4.2), 6 months (1.3, 2.3, 3.3, 4.3), and 2 years (1.4, 2.4, 3.4, 4.4). The grey bars represent the error bars.

Table 3. Similarity values of DGGE profiles of human faecal samples obtained at two different time points from 12 healthy subjects (baseline and 6 months later) with primers specific to the *Clostridium coccoides–Eubacterium rectale* (Erec) group of clostridia and with primers that target predominant microbiota in addition to proportions of the Erec group as quantified with the TRAC technique

		Mean ± SD	Range
Qualitative DGGE analysis			
With Erec primers			
RNA-based similarity (%)*	Baseline vs. 6 months	85.5 ± 10.6	55.5–95.8
DNA-based similarity (%)*	Baseline vs. 6 months	88.2 ± 5.8	75.4–94.7
RNA-based diversity†	Baseline	13.3 ± 2.3	10–16
RNA-based diversity†	6 months	13.8 ± 1.7	12–17
DNA-based diversity†	Baseline	14.3 ± 1.6	12–17
DNA-based diversity†	6 months	14.3 ± 1.9	12–18
With universal bacterial primers			
RNA-based similarity (%)*	Baseline vs. 6 months	84.2 ± 10.0	68.2–92.4
DNA-based similarity (%)*	Baseline vs. 6 months	83.4 ± 13.4	58.8–96.7
RNA-based diversity†	Baseline	18.5 ± 3.2	12–25
RNA-based diversity†	6 months	19.0 ± 2.0	16–22
DNA-based diversity†	Baseline	32.9 ± 7.4	24–51
DNA-based diversity†	6 months	31.5 ± 5.9	24–42
Quantitative TRAC analysis			
Erec (%)‡	Baseline	44.7 ± 16.0	17.7–71.2
Erec (%)‡	6 months	46.0 ± 10.1	35.1–66.9
ΔErec (%)§		9.3 ± 7.9	0.2–22.7

*Similarity values were counted with BioNumerics 4.01 software using Pearson correlation. Amplicons with a total surface area of at least 1% were included in the similarity analysis.
†Diversity is presented as the number of amplicons detected by the BioNumerics 4.01 software.
‡Percentage of bacteria that hybridized with the Erec482 probe as determined in triplicate.
§Difference in proportions (%) between the two sampling points.

the results obtained from our hybridization studies – one sample contained a high percentage of Erec-group bacteria and the other a low percentage. Our results showed that the amount of bacteria belonging to the Erec group was not related to the diversity of that same bacterial group. One sample (subject 4, baseline sample) containing 71% of Erec-group bacteria had 15 DGGE bands representing 21 OTUs, whereas another sample containing 27% of Erec-group

bacteria (subject 6, baseline sample) had 16 DGGE bands representing 22 OTUs.

The Erec group was found to be temporally as stable or unstable as the predominant bacteria in this study. In the 2-year stability study, the DGGE profiles of two subjects (1 and 3) were unstable/rather unstable, and two subjects (2 and 4) had minor differences with both predominant bacterial DGGE and Erec DGGE. In the shorter follow-up study (6 months) nearly all of the profiles that were unstable/rather unstable with either RNA- or DNA-based (or both) Erec DGGE were also unstable with predominant bacterial DGGE. However, the hybridization-detected proportional stability was not found to correlate with the DGGE-based stability. Five subjects showed instability in the proportion of the Erec group as detected with TRAC, whereas the DGGE profiles of only two of these subjects were found to be rather unstable. This finding further addresses the above-discussed difference between the proportion and diversity of a given bacterial group.

We found considerable intraindividual diversity as well as uniqueness of the predominant faecal bacterial population with both DNA-based (24–51 amplicons per sample) and RNA-based (12–25 amplicons per sample) PCR-DGGE analyses. The same intraindividual diversity was not observed in Erec-group DGGE profiles (12–18 amplicons per DNA sample; 10–17 amplicons per RNA sample), even though this group comprised the majority of the microbiota in some individuals according to the hybridization results. However, since it is possible to detect only those bacteria that constitute over 1% of the total population with DGGE (Muyzer *et al.*, 1993), we were able to detect the majority of the Erec-group bacteria, as can be seen from our clone-library results. The interindividual uniqueness of Erec profiles was not as clear as was the inter-individual uniqueness of the predominant bacterial profiles. Regardless of this, samples of a single subject clustered together in most cases. In addition, the clone libraries of the two subjects (4 and 6) indicated that, even though the DGGE profiles resembled each other and contained several similar OTUs, less than 20% of their sequences were identical.

RNA-based DGGE profiles of the predominant microbiota contained significantly fewer bands than the DNA-based DGGE profiles in this study. The differences were seen in all parts of the denaturing gradient. A clear difference between the DNA- and RNA-derived predominant faecal bacterial populations has also been reported by Tannock *et al.* (2004). However, this was not observed as commonly in our Erec-group DGGE profiles. Most of the intraindividual DNA- and RNA-based profiles were quite similar (10 out of 12 subjects).

In conclusion, the newly developed PCR-DGGE and RT-PCR-DGGE methods for the Erec group proved to be specific and useful in studying the diversity and stability of the human faecal microbiota. The DGGE profiles of all 12 healthy adults studied were diverse. In addition, the developed DGGE method proved to be a valuable tool for the direct assessment of the stability of the Erec group, demonstrating stability in most of the subjects studied.

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ARTICLE III

Diversity and temporal stability of fecal bacterial populations in elderly subjects consuming galacto-oligosaccharide containing probiotic yoghurt

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Diversity and temporal stability of fecal bacterial populations in elderly subjects consuming galacto-oligosaccharide containing probiotic yoghurt

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Abstract

Denaturing gradient gel electrophoresis was applied to study the effect of 3-week consumption of probiotic yoghurt containing galacto-oligosaccharides (GOS) on intra-individual diversity and temporal stability of predominant bacterial, bifidobacterial, *Lactobacillus*-group, and Erec-group *Eubacterium rectale–Clostridium coccoides* fecal populations of elderly subjects. Diversity and temporal stability of the selected bacterial groups of elderly subjects were compared with those obtained from younger adults in our previous studies. In the present study, GOS-yoghurt consumption did not significantly affect diversity or temporal stability of the selected bacterial groups. On average, the Erec-group and the predominant bacterial and bifidobacterial populations remained almost stable, and the *Lactobacillus*-group was unstable in elderly subjects. No differences were found between elderly and younger adults in temporal stability of studied bacterial populations. However, the difference in diversity of predominant bacterial population and Erec-group bacteria was significantly higher in elderly subjects of this study as compared with younger adults from our previous studies.

Keywords: Elderly; Fecal bacteria; DGGE; Yoghurt; GOS

1. Introduction

It was shown several decades ago that the gastrointestinal microbiota evolves with age (Gorbach, Nahas, Lerner, & Weinstein, 1967). As life expectancy in the western world has rapidly risen, interest in the gastrointestinal microbiota of elderly subjects has increased. Asian elderly subjects have been reported to have a lower level of bifidobacteria and higher levels of clostridia, lactobacilli, streptococci, and Enterobacteriaceae in culture-based studies (Benno et al., 1989; Mitsuoka, 1992). In European culture-based and molecular biology based studies, however, results have been partly contradictory (He, Harmsen, Raangs, & Welling, 2003; Hopkins, Sharp, & Macfarlane, 2001, 2002; Mueller et al., 2006; van Tongeren, Slaets, Harmsen, & Welling, 2005; Woodmansey, McMurdo, Macfarlane, & Macfarlane, 2004). Moreover, the difference in several bacterial groups between elderly and younger adults has been shown to be dependent on geographical location (Mueller et al., 2006). The number of bifidobacteria were found to be lower in elderly subjects compared with younger adults in European studies (Hopkins et al., 2001, 2002; Mueller et al., 2006; van Tongeren et al., 2005; Woodmansey et al., 2004).

Bifidobacteria are generally considered beneficial for health, and have been widely used, both individually and in combination with lactobacilli, in probiotic foods and products to increase the probiotic content in the human gastro-intestinal tract (Salminen, Deighton, Benno, &

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Gorbach, 1998). The absence of bifidobacteria, or their low numbers in the elderly, may have metabolic and health consequences for the host, affecting immune system function and a multiplicity of other functions, e.g., synthesis of vitamins and protein, and supplementation in digestion and absorption (Hopkins et al., 2001; Mitsuoka, 1992). In addition, bifidobacteria are involved in colonization resistance in the bowel (Hopkins et al., 2001). The number of bifidobacteria may be increased in the gut either by continuous supplementation of probiotic bifidobacteria or by adding prebiotics to food products. Prebiotics are non-digestible food ingredients that positively affect the host by selectively stimulating the growth, activity, or both of one or a limited number of beneficial bacterial species already resident in the colon (Gibson & Roberfroid, 1995). The prebiotics that currently fulfill the definition criteria are fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and lactulose (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004). GOS, derived from lactose in milk, have received less attention than the other prebiotics, although they are considered to have bifidogenic effects in humans. GOS is a collective term for a group of semi-synthetic non-digestible carbohydrates made using galactosidases as catalysts. GOS contain zero to one glucose units and one to six galactose units bound to each other by different glycosidic bonds (β1–2, β1–3, β1–4, β1–6). During production of GOS, a mixture of GOS of different chain length are formed (Alander et al., 2001; Ito et al., 1993; Tannock et al., 2004).

The aim of this study was to evaluate the impact of yoghurt containing GOS on the diversity and temporal stability of predominant fecal bacterial population and selected bacterial groups—namely the clostridial cluster XIVa (*Eubacterium rectale–Clostridium coccoides*-group, called the Erec-group), bifidobacteria, and lactobacilli—of elderly subjects suffering from constipation. In addition, the diversity and temporal stability of selected bacterial groups of elderly subjects were compared with those of younger adults.

2. Materials and methods

2.1. Study design

A double-blind, placebo-controlled, randomized cross-over study was performed on 41 elderly subjects with self-reported constipation (10 male and 31 female subjects, 60–79 years of age; mean 68 years). All the subjects had either difficulties in defecation most of the time or defecated less than five times weekly. Additional inclusion criteria were: aged between 60 and 80 years and a successful completion of the Mini-Mental State questionnaire (Folstein, Folstein, & McHugh, 1975). The Barthel index (Mahoney & Barthel, 1965) was used to evaluate the functional capacity of the subjects, and all participants received the full score 100. The exclusion criteria for both groups were daily use of laxatives, other gastrointestinal disorders (celiac disease, inflammatory bowel disease, gastrointestinal tumors, diverticulitis, or irritable bowel syndrome), thyroid dysfunction, use of strong psychopharmaceuticals or opioids, in addition to use of antimicrobials during the preceding month. The subjects defecated into a plastic container, and placed the samples immediately after defecation into their own freezer (−20 °C). Within 1 week, the samples were retrieved from the subjects and placed into a −70 °C freezer to await further analysis.

All subjects gave their written informed consent to participate in the study. The Human Ethics Committee of the Joint Authority for the Hospital District of Helsinki and Uusimaa (HUS, Finland) approved the study protocol.

Schematic representation of the study design is presented in Fig. 1. During the first intervention, elderly subjects of the GOS group consumed GOS-yoghurt whereas elderly subjects of the placebo group consumed placebo-yoghurt. After a wash-out period, during which neither group consumed test yoghurts, the second intervention was started. During this intervention, the elderly subjects of the GOS group consumed placebo-yoghurt whereas the elderly subjects of the placebo group consumed GOS-yoghurt. The GOS were produced enzymatically in situ in condensed milk, which was then used to prepare the GOS-yoghurt. Both the GOS- and placebo-yoghurts were fermented using a commercial starter culture containing *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidofilus*, and *Bifidobacterium lactis* strains, and yoghurts were flavored with vanilla. The yoghurts were low in lactose (<1 g per 100 g yoghurt), which was confirmed by high-pressure liquid chromatography (HPLC) analysis. The test yoghurts were

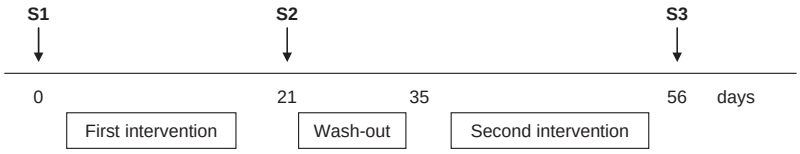

Fig. 1. Schematic representation of the study design. The arrows (S1–S3) indicate the time points for fecal sample collection (S1, baseline; S2, sample after 1st intervention; S3, sample after 2nd intervention). During the first intervention (sample S2), the GOS group consumed daily two portions (150 g per portion) of test yoghurt containing 5 g galacto-oligosaccharides (GOS) per portion and the placebo-group consumed daily two portions of test yoghurt (150 g per portion; no GOS addition). During the second intervention (sample S3), the GOS group consumed daily two portions of test yoghurt (150 g per portion; no GOS addition) and the placebo-group consumed daily two portions (150 g per portion) of test yoghurt containing 5 g galacto-oligosaccharides (GOS) per portion. During wash-out, elderly subjects of both groups consumed their normal diet, which did not include probiotic test yoghurt.

prepared at Valio Ltd. (Helsinki, Finland) and sent to the coordinator of the clinical trial as coded specimens. The daily GOS consumption for the GOS-yoghurt group was 10 g of GOS per day.

2.2. Polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) of predominant microbiota

DNA was extracted from 300 mg of fecal material using FastDNA Spin Kit for Soil (QBIOgene, Carlsbad, CA, USA), as described previously (Maukonen et al., 2006a). Partial 16S rRNA gene was PCR-amplified for the detection of predominant bacteria as described by Mättö et al. (2005) using primers U968-f+GC (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTA) and U1401-r (CGGTGTGTACAAGACCC) of Nübel et al. (1996). Bifidobacteria were PCR-amplified with primers Bif164-f (GGGTGGTAATGCCGGATG) and Bif662-GC-r (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCACCGTTACACCGGGAA) to evaluate the diversity and temporal stability of bifidobacteria according to Satokari, Vaughan, Akkermans, Saarela, and de Vos (2001a). The *Lactobacillus*-group, which comprises the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Weissella*, was amplified as described by Vanhoutte, Huys, De Brandt, and Swings (2004) using primers Lac1 (AGCAGTAGGGAATCTTCCA) and Lac2GC (CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCATTYCACCGCTACACATG; Walter et al., 2001). The Erec-group (Clostridial phylogenetic clusters XIVa; Collins et al., 1994) was PCR-amplified using primers Ccoc-f (AAATGACGGTACCTGACTAA; Matsuki et al., 2002) and Ccoc-r+GC (CGCCCGGGGCGCGCCCCGGGCGGGGGCGGGGGCACGGGGGGCTTTGAGTTTCATTCTTGCGAA), as described previously (Maukonen et al., 2006b).

The PCR products were separated in polyacrylamide gels with denaturing gradients of 38–60% (predominant bacteria and Erec-group), 45–55% (bifidobacteria), and 30–60% (*Lactobacillus*-group), where 100% is 7 M urea and 40% (v/v) deionized formamide, as described by Mättö et al. (2005). Similarity of the PCR-DGGE profiles of the samples obtained from a single subject at different sampling points was compared to evaluate the temporal stability of the investigated bacterial populations. The comparison of the profiles was performed by calculating the similarity percentage using BioNumerics software version 4.50 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Clustering was performed with Pearson correlation and the unweighted-pair group method. Amplicons with the total surface area of at least 1% were included in the similarity analysis. In addition to analysis of each different type of DGGE, a composite dataset including all the performed DGGE-analyses was created in the BioNumerics software.

2.3. Adult study group

In our previous studies we have analyzed adult microbiota diversity and stability (Maukonen et al., 2006a, 2006b; Saarela et al., 2007). The results from previous studies were used in the present study to evaluate the difference between diversity and temporal stability between elderly subjects and younger adults. All the previous younger adult control study groups consisted of healthy Finnish adults with normal intestinal balance (self-reported, no medical examination). The exclusion criteria for all control study groups were regular GI-tract symptoms, lactose-intolerance, celiac disease, and antimicrobial therapy during the last 2 months prior to each sampling time point. All the study groups were approved by the ethical committee of VTT Technical Research Centre of Finland, Espoo, Finland. All subjects gave their written informed consent for participation of the study in question.

The control study group of Maukonen et al. (2006a) consisted of 16 healthy adult volunteers (12 females, 4 males, age 26–63 years; mean 45 years) and the study group of Maukonen et al. (2006b) consisted of 12 healthy adult volunteers (nine females, three males, age 34–63 years; mean 47 years) who did not receive any additional supplementations to their normal Finnish diet. The control study group of Saarela et al. (2007) consisted of 10 healthy adult volunteers (nine females, one male, age 34–57 years; mean 41 years), who, after baseline sampling (the sample during normal Finnish diet, before the probiotic consumption started), were consuming a probiotic capsule containing *B. animalis* subsp. *lactis* Bb-12 and *L. acidophilus* LaCH-5, in addition to yoghurt starter bacteria *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*. The two subjects from study groups of Maukonen et al. (2006a) and Maukonen et al. (2006b), whose age was overlapping with the present study (age 60 or more) were excluded from the previous study groups.

Only baseline samples from all our previous healthy adult study groups (Maukonen et al., 2006a, 2006b; Saarela et al., 2007) were included in the comparison of bacterial diversity. For comparison of temporal stability of different bacterial groups, only the control study group of Saarela et al. (2007) was included, since this study group consumed the same probiotics and yoghurt starter bacteria for 2 weeks, as did the elderly groups of this study. In the previous studies, DGGE-analyses were performed and analyzed similarly as have been described in this study.

2.4. Statistical analysis

Mean and standard deviation were calculated for each experiment. Student's *t*-test (two-sample, assuming unequal variances) was used for the statistical analysis of the results obtained from a single DGGE-analysis. Multivariate analysis of variance (MANOVA) and principal component analysis (PCA), included in the BioNumerics

software (Applied Maths BVBA), were used for statistical analysis of the composite datasets. In addition, discriminant analysis including similarity between baseline and first intervention (Fig. 1; from all different DGGE analyses) and difference in band numbers (also from all different DGGE analyses) was performed with SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Diversity and temporal stability of the predominant bacteria, Erec-group, Lactobacillus-group, and bifidobacteria of elderly subjects

A carry-over effect was observed after the wash-out period (Surakka et al., unpublished data). A carry-over effect is such an effect that "carries over" from one treatment period to another. Whenever study participants perform in more than one treatment period, there is a possibility of carry-over effects. When using a cross-over design, one should test for possible carry-over by statistical methods. If carry-over is detected, as was unfortunately the case in the current trial in regard to, for example, fecal short-chain fatty acids, it is not advisable to analyze the data from the second treatment period. Therefore, only results which were obtained after the first intervention period (Fig. 1) are included in the results and in the statistical analysis.

3.2. Predominant bacteria

DGGE analysis targeted to the predominant bacterial population showed considerable intra-individual diversity as well as uniqueness of fecal microbiota before and after intervention (Fig. 2A). The intra-individual fecal samples clustered together in all of the cases (one distinct cluster per subject), irrespective of GOS- or placebo-yoghurt administration. Predominant fecal microbiota was temporally rather stable after both placebo (mean similarity 81.6 ± 10.5%) and GOS-yoghurt consumption (mean similarity 80.9 ± 11.9%) (Table 1). In addition, the number of amplicons after GOS- and placebo-yoghurt consumption was similar to the number of amplicons at the baseline (Table 2).

3.3. Erec-group

The intra-individual diversity and uniqueness of Erec-group DGGE in the fecal samples were similar to the case of predominant bacteria (Fig. 2B). Erec-group bacteria remained temporally mostly stable after both placebo (mean similarity 91.1 ± 6.1%) and GOS-yoghurt consumption (mean similarity 87.7 ± 7.9%) (Table 1). In addition, the number of amplicons after GOS- and placebo-yoghurt consumption was similar to the number of amplicons at the baseline (Table 2).

3.4. Lactobacillus-group

Lactobacillus-group targeted DGGE analysis showed intra-individual diversity as well as uniqueness of *Lactobacillus* microbiota before and after intervention (Fig. 2C). The intra-individual fecal samples clustered together in only 11 out of 37 subjects (one distinct cluster per subject, six placebo subjects and five GOS subjects, data not

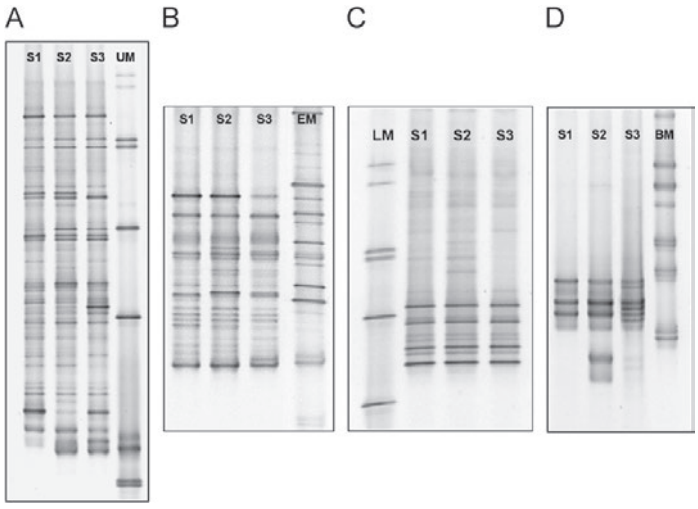

Fig. 2. Fecal denaturing gradient gel electrophoresis (DGGE) profiles of one elderly subject (placebo group, male, age 76 years) as detected with predominant bacterial polymerase chain reaction (PCR)–DGGE (A), *Clostridium coccoides–Eubacterium rectale*-group specific PCR-DGGE (B), *Lactobacillus*-group PCR–DGGE (C), and bifidobacteria-specific PCR–DGGE (D). The samples were obtained from fecal samples at time points baseline (S1), 3 weeks placebo yoghurt consumption (S2), and 3 weeks galacto-oligosaccharide (GOS) yoghurt consumption after 2 weeks wash-out period (S3); UM, universal marker; EM, Erec-marker; LM, lactobacilli-marker; BM, bifidobacteria-marker.

Table 1
Temporal stability (= similarity values) of denaturing gradient gel electrophoresis (DGGE) profiles of human fecal samples obtained at baseline and after consumption of test yoghurt with or without GOS supplementation from 41 elderly subjects with primers specific to *Clostridium coccoides–Eubacterium rectale* (Erec) group of clostridia, *Lactobacillus*-group, and bifidobacteria, in addition to primers that target predominant bacterial microbiota[a]

Analysis	Similarity (%)[b] (baseline vs. 1st intervention)			Similarity (%)[a] in adults[c]
	GOS	Placebo	All together[d]	
Predominant bacteria				
Mean ± SD	80.9 ± 11.9	81.6 ± 10.5	81.2 ± 11.2	81.0 ± 7.6
Range	51.4–95.5	58.6–94.9	51.4–95.5	70.0–90.6
Erec-group of clostridia				
Mean ± SD	87.7 ± 7.9	91.1 ± 6.1	89.1 ± 7.3	92.3 ± 4.7
Range	69.7–96.6	73.9–96.9	69.9–96.9	81.6–97.5
Lactobacillus-group				
Mean ± SD	59.1 ± 25.6	67.2 ± 28.3	62.6 ± 26.7	68.2 ± 11.9
Range	0.0–98.3	8.1–98.8	0.0–98.8	50.0–85.7
Bifidobacteria				
Mean ± SD	82.3 ± 21.7	82.0 ± 19.8	82.2 ± 20.6	87.1 ± 10.8
Range	0.5–99.3	17.3–97.8	0.5–99.3	68.2–97.8

[a]Temporal stability of DGGE profiles from an adult study group is also presented for the above-mentioned bacterial groups.
[b]Similarity values were counted with BioNumerics 4.50 software. Amplicons with the total surface area of at least 1% were included in the similarity analysis.
[c]Similarity values were counted from a healthy Finnish adult study group, which was consuming *Bifidobacterium animalis* subsp. *lactis* Bb-12 and *Lactobacillus acidophilus* LaCH-5, in addition to yoghurt starter bacteria *L. delbrueckii* subsp. *bulgaricus*, and *Streptococcus thermophilus* (Saarela et al., 2007; Maukonen et al., unpublished results).
[d]All together = GOS-group + placebo-group.

Table 2
Diversity of denaturing gradient gel electrophoresis (DGGE) profiles of human fecal samples obtained at baseline and after first intervention from 41 elderly subjects with primers specific to the Erec group of clostridia, *Lactobacillus*-group, and bifidobacteria, in addition to primers that target predominant bacterial microbiota[a]

Analysis	Diversity (number of bands)[b] in elderly subjects			Difference in diversity[c] (between 1st intervention and baseline)		Diversity (number of bands)[b] in adults[d]
	Baseline	GOS	Placebo	GOS	Placebo	
Predominant bacteria						
Mean ± SD	42.1 ± 5.0*	42.0 ± 4.6	43.6 ± 5.0	0.14 ± 3.27	0.65 ± 4.53	32.4 ± 4.7*
Range	35–57	35–51	35–52	−4–7	−8–11	24–40
Erec-group of clostridia						
Mean ± SD	20.7 ± 3.6*	20.8 ± 3.5	21.0 ± 3.0	−0.23 ± 2.81	0.29 ± 1.86	14.7 ± 2.1*
Range	14–28	14–26	16−26	−6–5	−3–3	11–19
Lactobacillus-group						
Mean ± SD	10.7 ± 4.0	11.9 ± 5.6	11.8 ± 2.8	0.57 ± 4.93	2.19 ± 2.69	8.2 ± 3.9
Range	2–17	5–30	7–18	−7–13	−2–7	4–17
Bifidobacteria						
Mean ± SD	8.6 ± 3.2	8.5 ± 2.7	9.5 ± 3.2	0.19 ± 1.03	0.71 ± 2.31	9.1 ± 2.8
Range	3–14	4–14	5–17	−2–2	−4–6	6–15

[a]The difference in diversity between baseline sample and a sample after first intervention is also presented. In addition, diversity of DGGE profiles from previous adult study groups is also presented for the above-mentioned bacterial groups.
[b]Diversity is presented as the number of bands detected by the BioNumerics 4.50 software (Applied Maths BVBA).
[c]Difference in diversity was calculated as (number of bands after 1st intervention)—(number of bands at baseline), therefore negative results indicate that there were less bands after the 1st intervention than at baseline, and positive results indicate that there were more bands after the 1st intervention than at the baseline. *Significant differences ($P<0.05$) in diversity between baseline samples of elderly subjects and adults.
[d]Diversity of DGGE-profiles in adults was calculated by combining the diversity of the baseline samples from healthy Finnish adult study groups of Maukonen et al. (2006a, 2006b, unpublished results) and Saarela et al. (2007). None of the subjects in the adult study groups had any supplementation to their normal Finnish diet at the time of the baseline sample.

shown). The subjects whose samples clustered together had an intra-individual temporal stability similarity value of (approx.) greater than 75%. All the other subjects had lower intra-individual temporal stability similarity values (data not shown). The samples of one subject did not amplify at all with the *Lactobacillus*-group PCR,

although the procedure was repeated with different amounts of DNA template for several times (data not shown).

The fecal lactobacilli population was temporally unstable (mean similarity 59.1±25.6%) after GOS-yoghurt consumption and temporally rather unstable (mean similarity 67.2±28.3%) after placebo consumption (Table 1). However, the difference in temporal stability between the study groups was not significant ($P = 0.19$). The number of amplicons after GOS- and placebo-yoghurt consumption was similar to the number of amplicons at the baseline (Table 2).

3.5. Bifidobacteria

The intra-individual diversity and uniqueness of bifidobacteria DGGE in the fecal samples were similar to the case of predominant bacteria and Erec-group bacteria (Fig. 2D). However, the intra-individual fecal samples clustered together in only 26/38 subjects (one distinct cluster per subject; data not shown). The subjects whose samples clustered together had an intra-individual temporal stability similarity value of (approx.) greater than 85%. The fecal bifidobacterial population was temporally mostly rather stable after both GOS-yoghurt consumption (mean similarity 82.3±21.7%) and after placebo-yoghurt consumption (mean similarity 82.0±19.8%) (Table 1). The number of amplicons after GOS- and placebo-yoghurt consumption was similar to the number of amplicons at baseline (Table 2).

3.6. Composite DGGE dataset

A composite dataset containing all different DGGE analyses was created in the BioNumerics software. According to a PCA plot, none of the sample groups (GOS, placebo, baseline) was distinguishable from the other groups (Fig. 3). However, the group of GOS samples was more concise (the samples more similar to each other) than the other sample groups (Fig. 3). The group of baseline samples was the most diverse (biggest ellipse; Fig. 3). Significant differences were not found in discriminant analysis or MANOVA on temporal stability and bacterial diversity on composite datasets.

3.7. Difference in diversity and temporal stability of the predominant bacteria, Erec-group, Lactobacillus-group, and bifidobacteria between elderly subjects and younger adults

In the comparison between elderly subjects of this study and younger adults of our previous studies (Maukonen et al., 2006a, 2006b; Saarela et al., 2007), only the placebo-yoghurt group was included in the comparison of temporal stability. For comparison of bacterial diversity, the baseline

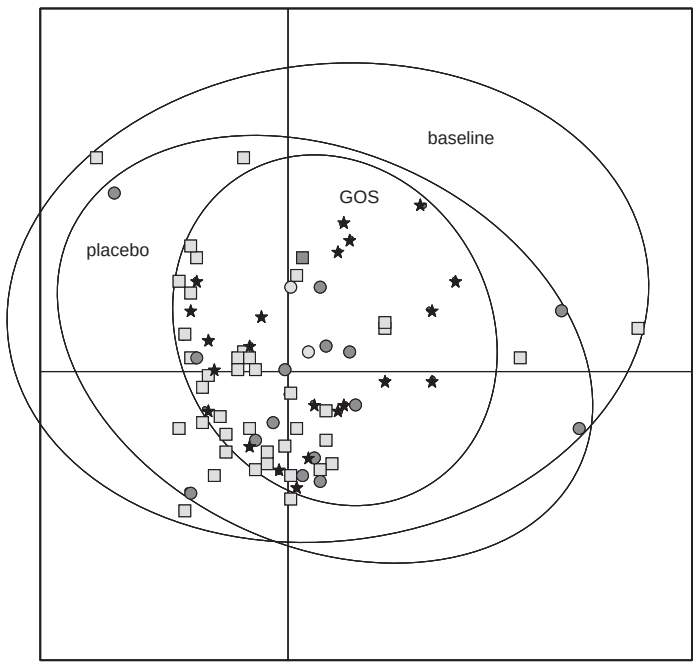

Fig. 3. A PCA plot of the composite denaturing gradient gel electrophoresis (DGGE) dataset (containing predominant bacterial, Erec-group, *Lactobacillus*-group, and bifidobacteria specific DGGE analyses) of baseline samples (light gray squares), samples after galacto-oligosaccharide (GOS) intervention (black stars), and samples after placebo intervention (dark gray dots).

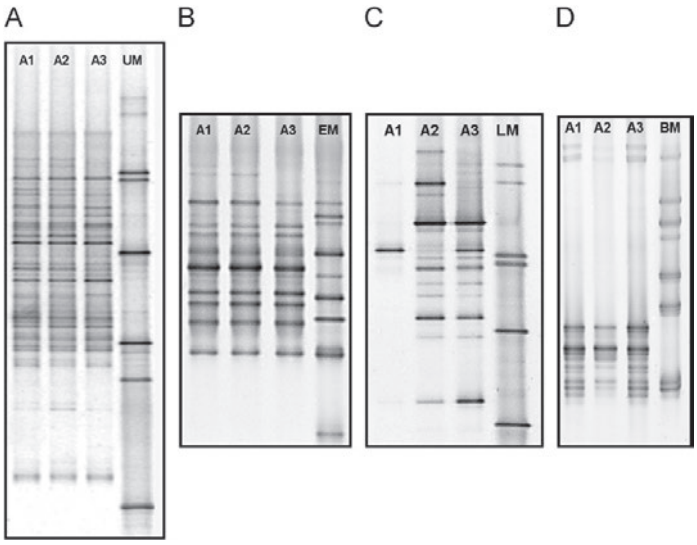

Fig. 4. Fecal denaturing gradient gel electrophoresis (DGGE) profiles of one healthy adult subject (female, 41 years old) as detected with predominant bacterial polymerase chain reaction (PCR)–DGGE (part of the study group of Saarela et al., 2007) (A); *Clostridium coccoides–Eubacterium rectale*-group specific PCR–DGGE (Maukonen et al., unpublished data) (B); *Lactobacillus*-group PCR–DGGE (Maukonen et al., unpublished data) (C); and bifidobacteria-specific PCR–DGGE (Maukonen et al., unpublished data) (D). The samples were obtained from fecal samples at time points baseline (A1), after 1-week probiotic capsule (containing *Bifidobacterium animalis* subsp. *lactis* Bb-12 and *Lactobacillus acidophilus* LaCH-5, in addition to yoghurt starter bacteria *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) consumption (A2), and 2 weeks probiotic capsule consumption (A3); UM, universal marker; EM, Erec-marker; LM, lactobacilli-marker; BM, bifidobacteria-marker.

samples of both GOS- and placebo-yoghurt groups were included. Temporal stability of studied bacterial groups did not differ between elderly and younger adults (Table 1). Diversity of predominant bacterial population was found to be significantly higher ($P<0.001$) in elderly subjects as compared to younger adults (42 ± 5 amplicons vs. 32 ± 5 amplicons, respectively; Table 2, Fig. 4A). In addition, diversity of Erec-group bacteria was significantly higher ($P<0.001$) in elderly subjects than in younger adults (21 ± 4 amplicons vs. 15 ± 2 amplicons, respectively; Table 2, Fig. 4B). Diversity of *Lactobacillus*-group of elderly subjects (11 ± 4 amplicons) was somewhat higher than that of younger adults (8 ± 4; $P=0.09$; Fig. 4C), whereas diversity of bifidobacteria was similar in both elderly subjects and younger adults (9 ± 3 amplicons; Table 2, Fig. 4D).

4. Discussion

The present study was designed as a cross-over study to minimize the effect of inter-individual variation on fecal microbiota. However, the 2-week wash-out period was not long enough for the present elderly subjects—probably due to their mild constipation—even though according to literature (Alander et al., 2001; Tannock et al., 2004) a 2-week wash-out period should have been sufficient for ingestion of GOS-supplemented foods. Therefore, we could not use the samples after the wash-out, which substantially reduced the number of subjects in both study groups. In addition, since our new study groups composed of different subjects, we cannot exclude possible minor changes to the investigated bacterial populations, which could have been seen at a person level, but could not be seen at a group level. The possible side effects of GOS were not investigated in this study, but according to earlier studies, the amount of GOS used in this study (10 g per day) is well-tolerated (Sairanen, Piirainen, Nevala, & Korpela, 2007; Teuri & Korpela, 1998). In addition, only one GOS concentration was used, but no differences regarding the bifidogenic effect were found between different GOS doses in an earlier study by Bouhnik et al. (2004). The effect of age on gastro-intestinal microbiota has been investigated mainly by enumeration techniques, either by cultivation, fluorescent in situ hybridization, or by real-time PCR (e.g., Bartosch, Fite, Macfarlane, & McMurdo, 2004; He et al., 2003; Hopkins et al., 2001, 2002; Mitsuoka, 1992; Mueller et al., 2006; van Tongeren et al., 2005; Woodmansey et al., 2004), whereas stability and diversity of fecal microbiota of elderly subjects has received less attention (Blaut et al., 2002; Hayashi, Sakamoto, Kitahara, & Benno, 2003; He et al., 2003; Hopkins & Macfarlane, 2002). We found only one study in which DGGE was applied to follow the stability and diversity of human fecal microbiota—predominant bacteria and bifidobacteria—during GOS ingestion (Tannock et al., 2004). However, the ages of the subjects (15 subjects) belonging to the study

group were not defined. Therefore, we are not able to make correct comparisons between our study group and that of Tannock et al. (2004).

We did not observe any changes in the predominant fecal bacterial microbiota of elderly subjects after the GOS-yoghurt intervention period. The similarity percentages remained similar in both placebo- and GOS-yoghurt groups. This has been observed also by Tannock et al. (2004), who found that GOS-containing biscuits had an effect in the RNA-derived predominant bacterial DGGE profiles but not in the DNA-derived DGGE profiles in New Zealand subjects. In addition, it has been shown on younger adults that the probiotics, which were included in the test yoghurt, did not have a great effect on the stability of the fecal predominant bacteria (Saarela et al., 2007). Species diversity in fecal microbiota increases with age (Blaut et al., 2002). The increase in predominant bacterial species diversity in elderly subjects was observed also in our study. The average number of amplicons found from elderly subjects was significantly higher than the average number of amplicons in younger adults in our previous studies (Maukonen et al., 2006a, 2006b; Saarela et al., 2007). In addition, the predominant bacterial DGGE profiles of elderly subjects in our study were different according to visual inspection as compared with DGGE profiles of younger adults in previous studies (Maukonen et al., 2006a, 2006b; Saarela et al., 2007). There were amplicons throughout the DGGE profile (from top to bottom) derived from feces of elderly subjects (Fig. 2A), whereas in the DGGE profiles of younger adults (Fig. 4A) there usually has been a small empty gap between the upper part of the DGGE gel (low-medium GC-content; most of the adult fecal microbiota) and the lower part of the gel (high GC-content; bifidobacteria and sulfate-reducing bacteria) (Maukonen et al., 2006a, 2006b; Saarela et al., 2007).

We did not find any difference in the stability or diversity of bacteria belonging to the Erec-group between the GOS- and placebo-yoghurt groups. The number of bacteria belonging to the Erec-group—as detected with FISH (fluorescent in situ hybridization)—has been shown to be lower in Dutch and Italian elderly subjects compared with younger adults (He et al., 2003; Mueller et al., 2006). On the contrary, the number of bacteria belonging to the Erec-group was higher in the French, German, and Swedish elderly subjects when compared with younger adults (Mueller et al., 2006). Since Finnish diets are close to Swedish diets, it could be expected that the number of bacteria belonging to the Erec-group would be higher in the elderly subjects than in the younger adults. We did not quantify the number of Erec-group bacteria, but we found significantly more amplicons from the elderly subjects in this study than we did from younger adults in our previous study (Maukonen et al., 2006b). In addition, Maukonen et al. (2006b) showed that each amplicon corresponded to a phylogenetic cluster, and therefore the diversity of fecal Erec-group of elderly subjects was higher than that of younger adults in our study. Contradictory to our findings, Hayashi et al. (2006) found as many operational taxonomic units (OTUs) of Erec-group bacteria in Japanese elderly and younger adults.

Since the number of bifidobacteria (Hopkins et al., 2001, 2002; Mitsuoka, 1992; Mueller et al., 2006; van Tongeren et al., 2005; Woodmansey et al., 2004) and the diversity of bifidobacterial population (Hopkins & Macfarlane, 2002; Woodmansey et al., 2004) have been reported to decrease with age, we wanted to study the effect of GOS-yoghurt on the bifidobacterial population. We did not find any difference in stability or diversity between the GOS- and placebo-yoghurt groups in agreement with Tannock et al. (2004) and Satokari, Vaughan, Akkermans, Saarela, and de Vos (2001b). In addition, the average number of amplicons that we found from elderly subjects was similar to the number of amplicons in healthy younger adults (Maukonen, unpublished results), which is not in agreement with the earlier culture based studies (Hopkins & Macfarlane, 2002; Woodmansey et al., 2004). We used a PCR–DGGE which did not target the ingested *B. animalis* subsp. *lactis* (Satokari et al., 2001a, 2001b), so this strain was not seen in the bifidobacterial DGGE-profiles, and therefore did not affect the diversity and temporal stability of the fecal bifidobacterial profiles.

Lactobacilli population has been shown to be temporally unstable in adults (Vanhoutte et al., 2004). We found the fecal lactobacilli population of elderly subjects to be unstable as well. Since the ingested *L. acidophilus* produced only a single band in the lactobacilli DGGE-profile, it did not constitute a considerable change of the similarity values between the samples. The lactobacilli population of the GOS-yoghurt group subjects was more unstable than that of the placebo-yoghurt group in our study. However, the difference was not statistically significant due to high standard deviation between subjects. Species diversity of fecal lactobacilli was similar in both study groups. In addition, we did not find a significant difference between the number of amplicons in elderly subjects and in younger adults of our previous studies (Maukonen, unpublished results).

5. Conclusions

Consumption of probiotic yoghurt containing galacto-oligosaccharide (GOS) for 3 weeks did not significantly affect the diversity or temporal stability of predominant bacterial, bifidobacterial, *Lactobacillus*-group or *Eubacterium rectale—Clostridium coccoides* group (Erec-group) fecal populations in elderly subjects. On average, the Erec-group bacteria remained stable, the predominant bacterial and bifidobacterial populations remained reasonably stable and the *Lactobacillus*-group was unstable in both study groups. Moreover, the difference in temporal amplicon diversity of the GOS-yoghurt group was smaller in all studied bacterial groups as compared to the placebo-yoghurt group.

We did not find any differences between elderly and younger adults in temporal stability of studied bacterial populations. However, the difference in diversity of predominant bacterial population and Erec-group bacteria was found to be significantly higher in elderly subjects as compared to younger adults.

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ARTICLE IV

Intra-individual diversity and similarity of salivary and faecal microbiota

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Intra-individual diversity and similarity of salivary and faecal microbiota

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In the present study, polyphasic analysis [cultivation, combined with the fingerprinting of individual isolates, and denaturing gradient gel electrophoresis (DGGE)] was applied to study whether similar features concerning the diversity and temporal stability of selected bacterial groups could be detected intra-individually in two different niches – the oral cavity and the colon – from ten adult volunteers consuming probiotics. The predominant bacterial microbiota, *Clostridium coccoides–Eubacterium rectale* group and bifidobacterial populations, were generally stable in salivary and faecal samples, with the greater diversity seen in faeces. Furthermore, different species predominated at the two different sites. *Lactobacillus* group DGGE profiles were unstable, yet the intra-individual profiles from faecal and salivary samples collected at the same time resembled each other. The ingested probiotic product did not affect the stability of the bacterial groups studied. The culture-based analysis showed that most subjects harboured identical indigenous *Lactobacillus* genotypes in saliva and faeces (*Lactobacillus rhamnosus*, *Lactobacillus gasseri*, *Lactobacillus paracasei* and *Lactobacillus plantarum* group). Thus, identical indigenous lactobacilli were able to inhabit both ends of the orogastrointestinal tract, whereas the composition of the other bacterial groups studied varied between the two sites.

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INTRODUCTION

Both ends of the orogastrointestinal tract of humans have an abundant microbiota dominated by anaerobically growing bacteria (Berg, 1996). The number of bacteria in the oral cavity is about 10^{11} (g wet weight dental plaque)$^{-1}$ and 10^{8}–10^{9} (ml saliva)$^{-1}$ (culturable bacteria; Nisengard & Newman, 1994; Li *et al.*, 2005), whereas in faeces the corresponding figure is $6–9 \times 10^{10}$ (g wet weight)$^{-1}$ (detected with molecular techniques; Thiel & Blaut, 2005). Although the same bacterial genera can be found in oral and colonic samples, to the best of our knowledge only one intra-individual comparative study of bacterial populations (in this case lactobacilli) of these two sampling sites has been performed previously in humans (Dal Bello & Hertel, 2006).

The faecal microbiota is dominated by the *Clostridium coccoides–Eubacterium rectale* (Erec) group, *Clostridium leptum* group and *Bacteroidetes* group (Franks *et al.*, 1998; Suau *et al.*, 1999; Sghir *et al.*, 2000; Eckburg *et al.*, 2005), which account for over 70 % of faecal bacteria (Sghir *et al.*, 2000). The bacteria belonging to the Erec group (clostridial cluster XIVa) (Collins *et al.*, 1994) comprise 10–59 % of the total faecal bacteria depending on the detection method used (Franks *et al.*, 1998; Suau *et al.*, 1999; Sghir *et al.*, 2000; Eckburg *et al.*, 2005; Maukonen *et al.*, 2006a). *Bifidobacterium* and *Lactobacillus*, although found in most subjects, comprise smaller populations among faecal bacteria in adults (bifidobacteria, 1–5 %; lactobacilli, <1–2 %; Franks *et al.*, 1998; Sghir *et al.*, 2000). The oral cavity contains different micro-environments (cheeks, palate, tongue, tooth surfaces, gingival areas and saliva), each with their own microbiota (Aas *et al.*, 2005). The salivary microbiota reflects a mixture of bacteria washed off from the various surfaces, especially from the tongue (Nisengard & Newman, 1994). A large diversity of bacteria has been detected in the oral cavity, including low-mol% G+C-content Gram positive organisms (e.g. streptococci and *Clostridia* group), fusobacteria, actinobacteria, different proteobacteria, *Prevotella*, *Porphyromonas*, *Bacteroides* and spirochaetes. Members of the clostridial cluster XIVa have occasionally been detected in oral samples (Paster *et al.*, 2001). In the oral cavity, both bifidobacteria and lactobacilli can be detected, but their occurrence is reversed compared with faeces: lactobacilli are a common finding in the oral cavity, whereas bifidobacteria are detected less frequently.

The aim of this study was to investigate whether similar features concerning the diversity and temporal stability of the predominant microbiota and selected bacterial groups

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Abbreviations: DDGE, denaturing gradient gel electrophoresis; Erec, *Clostridium coccoides–Eubacterium rectale*; RAPD, randomly amplified polymorphic DNA.

– namely the clostridial cluster XIVa, bifidobacteria and lactobacilli – could be detected intra-individually in two different niches: the oral cavity and the colon. Special focus was also put on the species distribution of the genera *Lactobacillus* and *Bifidobacterium* at these two sites.

METHODS

Sample handling. Faecal and salivary (5 ml saliva collected with paraffin stimulation) samples were collected from ten adult volunteers (nine females, one male, aged 34–57 years) at three sampling time points (at baseline before probiotic consumption and after 1 and 2 weeks of probiotic consumption). The subjects consumed a commercial probiotic capsule preparation (Trevis, 54 capsules; Ipex Medical) according to the manufacturer's instructions (three capsules each day) for 2 weeks. Trevis capsules contain a mixture of *Lactobacillus acidophilus* LaCH-5, *Bifidobacterium animalis* subsp. *lactis* Bb-12 and yoghurt starter bacteria, totalling 10^9–10^{10} organisms per capsule. The main recruiting criterion was a normal intestinal balance (absence of repeating and/or persisting gastrointestinal symptoms). The exclusion criteria were regular gastrointestinal tract symptoms, lactose intolerance, coeliac disease and antimicrobial therapy during the 2 months prior to the study. The study was approved by the ethical committee of the VTT Technical Research Centre of Finland. All subjects gave written informed consent for participation in the study.

Part of the faecal sample was transferred to Cary–Blair transport medium (Atlas, 1997) and part of the salivary sample to VMGA III medium (Rams *et al.*, 1990), and these were analysed by culture within 1–2 days. The stability of the studied bacterial groups in the transport medium was confirmed by culturing four samples immediately after sampling and after storage for 2 days in the appropriate transport medium. The rest of the samples were frozen at −70 °C for DNA-based analyses.

Culture-based analysis

The samples were serially diluted in pre-reduced peptone saline containing 0.5 g L-cysteine/HCl l^{-1} (pH 6.3; Merck) and plated on culture medium in an anaerobic workstation (Don Whitley Scientific). The following culture media and incubation conditions were used (incubation at 37 °C): supplemented Brucella blood agar (Tammer-Tutkan Maljat) for anaerobes (anaerobic incubation for 7 days), sheep blood agar for aerobes (Tammer-Tutkan Maljat) (aerobic incubation for 4 days) and Beerens agar for bifidobacteria (Beerens, 1991) (anaerobic incubation for 4 days). For the detection of *B. animalis* subsp. *lactis* Bb-12, the samples were also acid pre-treated (Alander *et al.*, 2001). Rogosa agar was used for the detection of lactobacilli (anaerobic incubation for 3 days). For the detection of *L. acidophilus* LaCH-5, the Rogosa plates were incubated micro-aerophilically. Beerens and Rogosa agars were also used with tetracycline supplementation (8 μg ml^{-1}), as *B. animalis* subsp. *lactis* Bb-12 is intermediately resistant to tetracycline.

Isolates representing all of the different potential *Lactobacillus* and *Bifidobacterium* colony morphologies, or, in the case of uniform colony morphology, random isolates, were collected from Beerens- and Rogosa-based media (five isolates from each medium where possible) for comparison of the species distribution in saliva and faeces.

Fingerprinting of bifidobacteria and lactobacilli by randomly amplified polymorphic DNA (RAPD). A total of 587 isolates from Beerens medium were analysed by RAPD using primer OPA-2 (5′-TGCCGAGCTG-3′) and 574 isolates from Rogosa were analysed by RAPD using primer OPA-3 (5′-AGTCAGCCAC-3′) as described previously (Alander *et al.*, 2001). The RAPD fingerprints of faecal and salivary isolates were compared with those of the *B. animalis* subsp. *lactis* Bb-12 and *L. acidophilus* LaCH-5 strains by visual inspection. All types that looked different to the ingested probiotic strains were selected for further characterization.

Partial 16S rRNA gene sequencing. Isolates representing different RAPD types were identified by partial 16S rRNA gene sequencing. Beerens isolates were amplified with Bif164-f (5′-GGGTGGTAATG-CCGGATG-3′) and Bif662-GC-r (5′-CGCCCGCCGCGCGCGGCG-GGCGGGGCGGGGGCACGGGGGGCCACCGTTACACCGGGAA-3′) as described by Satokari *et al.* (2001a). Rogosa isolates were amplified with BSF8/20 (5′-AGAGTTTGATCCTGGCTCAG-3′) and BSR1541/20 (5′-AAGGAGGTGATCCAGCCGCA-3′) (Wilmotte *et al.*, 1993). The lactobacilli PCR mixture contained 0.2 mM each dNTP, 0.2 μM primer and 3 U Dynazyme II DNA polymerase in 1 × Dynazyme buffer. PCR amplification was carried out in a thermocycler (UnoII, Biometra; 35 cycles with an annealing temperature of 56 °C). The amplification products were checked, purified and further sequenced with primers Bif164-f or BSF8/20 as described previously (Maukonen *et al.*, 2006b).

PCR-denaturing gradient gel electrophoresis (DGGE) analysis. DNA was extracted as described by Maukonen *et al.* (2006b). Part of the 16S rRNA gene (V_6–V_8 hypervariable region) was PCR amplified for the detection of predominant bacterial microbiota using primers U968-f+GC (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGG-GCACGGGGGGAACGCGAAGAACCTTA-3′) and U1401-r (5′-CGGTGTGTACAAGACCC-3′) (Nübel *et al.*, 1996), as described by Mättö *et al.* (2005). Primers Bif164-f and Bif662-GC-r were used to evaluate the diversity and temporal stability of bifidobacteria according to Satokari *et al.* (2001a). Primers Bif164mod-f (5′-GGGTGGTAATACCGGATG-3′) and Bif662-GC-r (Satokari *et al.*, 2001b) were used for the detection of *B. animalis* subsp. *lactis* Bb-12. The *Lactobacillus* group, which comprises the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Weissella*, was amplified using primers Lac1 (5′-AGCAGTAGGGAATCTTCCA-3′ and Lac2GC (5′-CGCCC-GCCGCGCCCCGCGCCCGGCCCGCCG CCCCCGCCCCATTYCA-CCGCTACACATG-3′) (Walter *et al.*, 2001), as described by Vanhoutte *et al.* (2004). The Erec group was PCR amplified using primers Ccoc-f (5′-AAATGACGGTACCTGACTAA-3′; Matsuki *et al.*, 2002) and Ccoc-r+GC (5′-CGCCCGGGGCGCGCCCCGGGCGG-GGCGGGGGCACGGGGGGCTTTGAGTTTCATTCTTGCGAA-3′) according to Maukonen *et al.* (2006b).

PCR products were separated by polyacrylamide gels with a denaturing gradient of 38–60 % (predominant bacterial microbiota and Erec group), 45–55 % (bifidobacteria) or 30–60 % (*Lactobacillus* group) [where 100 % is 7 M urea and 40 % (v/v) deionized formamide] as described by Mättö *et al.* (2005). *L. acidophilus* LaCH-5 was added as a probiotic control lane to each *Lactobacillus* group-specific DGGE. Comparison of the PCR-DGGE profiles was performed as described previously (Maukonen *et al.*, 2006b).

Statistical analysis. Means ± SD were calculated for each experiment. Results with microbial numbers below the detection limit (log 4 for all microbial groups) were excluded from statistical analysis using Student's *t*-test (two-sample test assuming unequal variances).

RESULTS AND DISCUSSION

In the present study, both the culture-based approach (combined with fingerprinting of individual isolates) and PCR-DGGE were applied to study the intra-individual diversity and temporal stability of microbiota at two different sites, the oral cavity and the colon. To the best of our knowledge, there is only one previous study where the

oral and faecal bacterial populations have been compared intra-individually using molecular techniques (Dal Bello & Hertel, 2006). The probiotic product used in our study did not notably affect the stability of the predominant microbiota profile, Erec group profile or bifidobacterial profile (using non-modified primers that targeted only the intestinal bifidobacteria and not the ingested probiotic bifidobacteria), as the baseline DGGE profiles (before probiotic ingestion) were highly similar to those of the samples after 1 and 2 weeks of probiotic ingestion. All of these bacterial groups remained relatively stable in most of the subjects. Our study showed that, in the cases of predominant microbiota, bifidobacteria and Erec group bacteria, the faecal microbiota was more diverse than that in saliva. In addition, salivary and faecal DGGE profiles of these groups appeared to be substantially different. Interestingly, most subjects' faecal and salivary samples contained identical indigenous *Lactobacillus* species and genotypes.

Compliance was evaluated with culture-based techniques and DGGE analysis in this study. Probiotic strains were not found in any of the baseline samples, whereas RAPD types identical to *B. animalis* subsp. *lactis* Bb-12 were found from all faecal samples during probiotic ingestion (after 1 and 2 weeks). Ingested probiotic strains were not found in any of our salivary samples.

Diversity and temporal stability of the predominant bacterial microbiota, Erec group, *Lactobacillus* group and bifidobacteria as detected with DGGE

Predominant bacterial microbiota

DGGE analysis targeted to the predominant microbiota showed intra-individual diversity as well as uniqueness of both faecal and salivary microbiota (Fig. 1a). After comparison of all faecal- and saliva-based profiles, faecal profiles and salivary profiles formed two distinct clusters, with individual-based subclusters (data not shown). Indeed, the DGGE profiles of faecal and salivary samples differed substantially (mean similarity 23.7 ± 7.1 %; Table 1). The predominant faecal microbiota was relatively stable temporally (mean similarity 83.3 ± 5.3 %) and the salivary microbiota was mostly stable (mean similarity 92.2 ± 2.5 %) during the study period (Table 2). This is consistent with previous work, which showed that the faecal microbiota is host specific and relatively stable temporally (Zoetendal *et al.*, 1998; Vanhoutte *et al.*, 2004; Maukonen *et al.*, 2006b). Likewise, in a recent study by Rasiah *et al.* (2005), it was shown that the predominant salivary microbiota remained stable in one subject for 7 years. We found a significant difference between the number of amplicons detected in the faecal and salivary profiles (faeces>saliva; $P<0.05$) (Table 2). Aas *et al.* (2005) showed that 20–30 different predominant species were found from most oral sites, utilizing 16S rRNA gene clone libraries. Our universal DGGE results are in accordance with this previous work.

Erec group

DGGE analysis targeted to the Erec group showed intra-individual diversity as well as uniqueness of both faecal and salivary microbiota (Fig. 1b). Erec profiles of faecal samples clustered together according to subject, whereas salivary samples clustered according to subject in eight cases (data not shown). The similarity between faecal samples and salivary samples from the same subject at a given time point was very low or non-existent (mean similarity

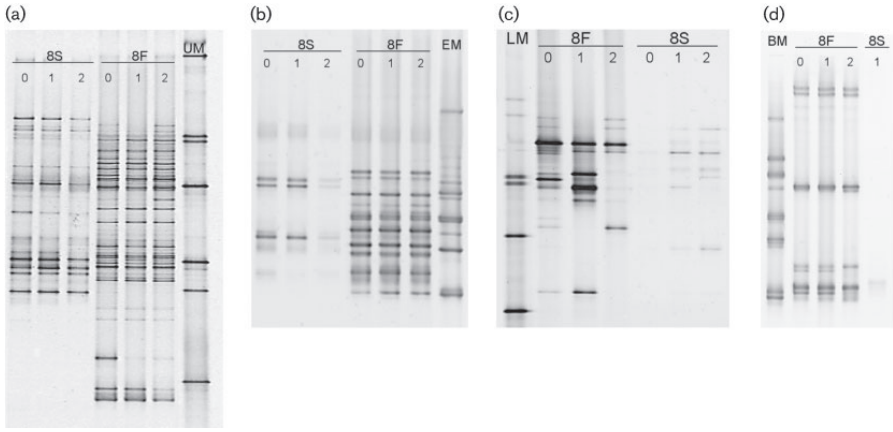

Fig. 1. Faecal and salivary DGGE profiles of a healthy subject. (a) Universal primers; (b) Erec group primers; (c) *Lactobacillus* group primers; (d) bifidobacteria primers. DNA was obtained from faecal samples (8F) and salivary samples (8S) at baseline (before probiotic consumption; lane 0), and after 1 and 2 weeks of probiotic consumption (lanes 1 and 2, respectively). BM, Bifidobacterial marker; EM, Erec group marker; LM, lactobacillus marker; UM, universal marker.

Table 1. Intra-individual similarity values between a faecal sample and a salivary sample at a given time point for a given subject from ten healthy subjects before and during probiotic consumption

Analysis	Similarity (%)*			
	Baseline†	1 week	2 weeks	Mean
Predominant bacteria‡				
Faeces vs saliva (mean ± SD)§	25.7 ± 10.9	22.5 ± 7.7	22.9 ± 8.7	23.7 ± 7.1
Range§	10.8–43.8	4.8–32.9	9.5–37.7	16.1–38.1
Erec group of clostridia‖				
Faeces vs saliva (mean ± SD)§	17.4 ± 10.0	15.7 ± 10.4	20.7 ± 17.8	17.9 ± 11.1
Range§	0.0–31.3	0.0–32.2	4.9–67.3	1.6–39.0
***Lactobacillus* group¶**				
Faeces vs saliva (mean ± SD)§	58.9 ± 18.3	60.2 ± 8.3	58.7 ± 17.0	58.6 ± 11.2
Range§	26.7–85.7	54.5–80.0	26.7–80.0	40.3–74.3
Bifidobacteria				
Faeces vs saliva (mean ± SD)§#	19.8 ± 13.2	23.9 ± 28.2	26.6 ± 32.7	26.4 ± 27.9
Range§	0.0–33.3	0.0–77.5	0.0–81.7	0.0–81.7

*Similarity values were analysed using BioNumerics 4.50 software. Amplicons with the total surface area of at least 1 % were included in the similarity analysis.

†Sample before 2 weeks of probiotic consumption.

‡Partial 16S rRNA gene (V_6–V_8 hypervariable region).

§The salivary and faecal samples were compared only intra-individually, that is, for example, a faecal sample of subject A taken at baseline was compared only with the salivary sample of subject A taken at baseline.

‖Clostridial phylogenetic cluster XIVa (Collins *et al.*, 1994).

¶*Lactobacillus* group was comprised the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Weissella*.

#Only half of the saliva samples gave a positive result after bifidobacteria-specific PCR.

17.9 ± 11.1 %; Table 1). Faecal samples of all subjects clustered together as one distinct cluster and salivary samples of all subjects clustered as another.

Maukonen *et al.* (2006b) showed that each band position in DGGE gels contained only one phylotype. We may therefore assume that the phylotypes present in the faeces and saliva are mostly different. Erec group bacteria were temporally stable in most cases (mean similarity: faeces 94 ± 2.9 %; saliva 90.5 ± 7.3 %) during our study period (Table 2), as has also been described previously (Maukonen *et al.*, 2006b). In this study, the Erec group diversity in salivary samples was significantly lower than in the faecal samples ($P<0.05$). Nonetheless, we found between 3 and 12 amplicons from each sample (Table 2), indicating that the diversity of the Erec group in saliva in some subjects may be slightly greater that that shown previously with other techniques (Downes *et al.*, 2001; Paster *et al.*, 2001).

Lactobacillus group

In this study, the DGGE profiles of the faeces- and saliva-derived *Lactobacillus* group were more similar to each other (mean similarity 58.6 ± 11.2 %; Table 1) than the faeces- and saliva-derived profiles of the other bacterial groups studied. In addition, faecal *Lactobacillus* group profiles of nearly all subjects were fairly unstable (mean similarity 69.1 ± 7.3 %; Table 2, Fig. 1c), whereas salivary *Lactobacillus* group profiles remained rather stable (mean similarity 89.8 ± 7.5 %; Table 2). Based on the comparisons made between different sampling points (baseline without probiotic consumption vs samples during probiotic consumption), the ingested *L. acidophilus* LaCH-5 strain, which was seen in the faecal lactobacilli DGGE profile during probiotic ingestion, does not alone explain the instability.

As the LaCH-5 strain produced only a single band, it did not contribute to a considerable change in the similarity values between the samples. In the salivary samples, there were no amplicons that were identical to those of *L. acidophilus* LaCH-5. In BioNumerics analysis, the lack of a stable and host-specific *Lactobacillus* group population resulted in a complete lack of subject grouping in faecal samples and sample type grouping (faeces vs saliva). This lack of subject grouping has also been reported for faecal samples by Vanhoutte *et al.* (2004). The number of amplicons detected in our study was higher in faecal samples compared with salivary samples (Table 2).

Bifidobacteria

In this study, DGGE profiles of faecal samples of a given subject clustered together in nine out of ten subjects (data not shown). In these cases, the faecal bifidobacterial population remained fairly stable temporally (one subject was unstable) (Table 2, Fig. 1d), as has also been found by, for example, Satokari *et al.* (2001a). Only about half of the

Table 2. Intra-individual similarity values and diversity of DGGE profiles of human faecal and salivary samples obtained at three different time points from ten healthy subjects

Analysis	Similarity (%)*				Diversity†			
	0 vs 1	0 vs 2	1 vs 2	Mean	0 week	1 week	2 weeks	Mean
Predominant bacteria‡								
Faeces (mean ± SD)	84.3 ± 7.8	81.0 ± 7.6	84.7 ± 4.9	83.3 ± 5.3	33.7 ± 4.2	35.8 ± 4.4	35.1 ± 4.4	34.9 ± 4.0
Range	70.3–92.8	70.0–90.6	75.6–92.2	72.0–90.9	25–39	27–42	27–41	26.3–40.7
Saliva (mean ± SD)	92.1 ± 3.3	91.4 ± 2.5	93.2 ± 4.4	92.2 ± 2.5	23.9 ± 2.9	23.1 ± 2.2	23.8 ± 2.9	23.6 ± 2.4
Range	86.0–95.8	87.2–94.2	82.9–98.0	88.8–95.3	20–27	19–26	19–28	19.3–26.7
Erec group§								
Faeces (mean ± SD)	95.1 ± 2.8	92.3 ± 4.7	94.8 ± 2.0	94.0 ± 2.9	15.1 ± 2.4	15.5 ± 2.3	14.8 ± 1.9	15.1 ± 2.0
Range	89.9–98.6	81.6–97.5	90.0–97.3	87.1–96.9	11–19	13–19	12–19	12.3–18.7
Saliva (mean ± SD)	92.9 ± 7.7	86.7 ± 11.8	92.0 ± 9.2	90.5 ± 7.3	7.4 ± 2.1	7.4 ± 1.6	7.7 ± 2.0	7.5 ± 1.7
Range	73.8–98.2	66.5–98.0	70.0–99.4	77.2–98.0	3–11	5–10	5–12	4.7–9.7
***Lactobacillus* group‖**								
Faeces (mean ± SD)	70.0 ± 8.1	68.2 ± 11.9	70.7 ± 14.0	69.1 ± 7.3	12.0 ± 5.3	10.9 ± 2.8	11.0 ± 2.4	11.5 ± 3.0
Range	60.0–85.7	50.0–85.7	50.0–88.9	61.0–85.7	7–22	7–15	7–14	8.0–16.3
Saliva (mean ± SD)	92.2 ± 13.9	87.6 ± 11.0	89.5 ± 6.5	89.8 ± 7.5	7.0 ± 2.8	7.9 ± 3.1	7.0 ± 3.4	7.3 ± 2.9
Range	66.7–100	66.7–100	80.0–100	76.9–100	3–13	5–14	4–15	3.7–14.0
Bifidobacteria								
Faeces (mean ± SD)	94.0 ± 3.5	87.1 ± 10.8	82.3 ± 16.2	87.8 ± 9.6	9.1 ± 2.8	8.8 ± 2.4	9.1 ± 3.2	9.0 ± 2.6
Range	90.9–99.6	68.2–97.8	52.5–98.1	70.7–97.1	6–15	7–15	4–14	5.3–14.7
Saliva (mean ± SD)¶	84.7 ± 17.9	97.9 ± 2.0	89.2 ± 15.7	86.4 ± 16.0	4.5 ± 1.9	5.8 ± 1.5	4.0 ± 1.7	4.4 ± 1.4
Range	62.3–98.9	95.5–99.7	65.6–98.0	62.3–98.0	2–7	4–8	2–7	3.0–6.7

*Similarity values were calculated using BioNumerics 4.50 software. Amplicons with a total surface area of at least 1 % were included in the similarity analysis. 0, baseline (before probiotic consumption); 1, 1 week of probiotic consumption; 2, 2 weeks of probiotic consumption.
†Diversity is presented as the number of bands making up >1 % of the total profile as detected by the BioNumerics 4.50 software.
‡Partial 16S rRNA gene (V_6–V_8 hypervariable region).
§Clostridial phylogenetic cluster XIVa (Collins *et al.*, 1994).
‖*Lactobacillus* group was comprised the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Weissella.*
¶Only half of the salivary samples gave a positive result after bifidobacteria-specific PCR.

salivary samples (17/30 samples) gave a positive PCR result, although several additional variations to the PCR protocol were tested (data not shown). The salivary bifidobacterial populations were temporally stable in three subjects and unstable in one subject (a different subject from the one who had an unstable faecal bifidobacterial population; data not shown). Of the remaining six subjects, only one sample from three subjects and two samples from another subject produced a PCR product, thus not allowing temporal stability to be determined. The bifidobacterial profiles did not cluster according to sample type, even though the similarity between faecal and salivary samples collected at the same time was low. The faecal samples contained significantly more amplicons than the salivary samples at all sampling time points ($P<0.05$) (Table 2). As the PCR primers used for the DGGE-based diversity assessment had one base mismatch (Satokari *et al.*, 2001b) with the sequence of the ingested *B. animalis* subsp. *lactis* Bb-12, this strain was not seen in the bifidobacterial DGGE profiles and therefore did not affect the temporal stability of the faecal and salivary profiles. However, Bb-12 was detected in faecal samples from seven of the ten subjects using DGGE targeting *B. animalis* subsp. *lactis* Bb-12. There were no amplicons that migrated at identical positions to those of *B. animalis* subsp. *lactis* Bb-12 in any of the salivary samples.

Culture

Mean numbers of culturable bacteria on media without tetracycline at different sampling occasions are shown in Fig. 2. The number of culturable bacteria remained stable during probiotic consumption in both faecal and salivary samples. The number of culturable anaerobic bacteria was significantly higher in faeces (mean log 10.5 c.f.u. g^{-1}) than in saliva (mean log 8.3 c.f.u. g^{-1}) ($P<0.05$), whereas the number of culturable aerobic bacteria was significantly higher in saliva (mean log 7.8 c.f.u. g^{-1}) than in faeces (mean log 7.1 c.f.u. g^{-1}) ($P<0.05$). The numbers of both bifidobacteria and lactobacilli were significantly higher in

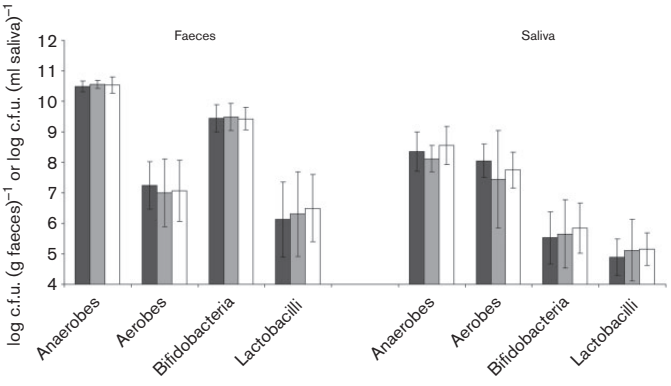

Fig. 2. Bacterial numbers (counts of bacteria growing on non-selective and bifidobacteria- or lactobacilli-selective culture media) in faecal and salivary samples of ten healthy volunteers. The samples were obtained at baseline before probiotic consumption (black bars), after 1 week of probiotic consumption (grey bars) and after 2 weeks of probiotic consumption (white bars).

faeces compared with saliva ($P<0.05$) (Fig. 2). The numbers of culturable bacteria found in salivary and faecal samples were in accordance with previous studies (Sanyal & Russel, 1978; Matsuki *et al.*, 1999; Tannock *et al.*, 2000).

Isolates with an identical RAPD type to the ingested *B. animalis* subsp. *lactis* Bb-12 strain were recovered from faecal samples but not from the salivary samples of all subjects during probiotic consumption (samples from 1 and 2 weeks), whereas isolates of *L. acidophilus* LaCH-5 strain were recovered from faecal samples of all subjects after 1 week of probiotic consumption and from nine out of ten subjects after 2 weeks.

From each subject, three to six indigenous salivary lactobacilli RAPD types and one to five indigenous faecal lactobacilli RAPD types were detected (Table 3). In addition, identical indigenous lactobacilli RAPD types were found in saliva and faeces for eight of the ten subjects (Table 3). These included *Lactobacillus rhamnosus*, *Lactobacillus gasseri*, *Lactobacillus paracasei*, *Lactobacillus plantarum* group (comprising *L. plantarum*, *Lactobacillus arizonensis* and *Lactobacillus pentosus*) and a *Lactobacillus* sp.

In a study by Dal Bello & Hertel (2006), *Lactobacillus* populations of salivary and oral samples of three subjects were compared by culture and RAPD typing in addition to DGGE. They found that *L. gasseri*, *L. paracasei*, *L. rhamnosus* and *Lactobacillus vaginalis* were most commonly detected among the predominant lactobacilli in the saliva and faeces of their three subjects. However, we only found *L. vaginalis* from one subject's saliva and none of the faecal samples of our subjects. *L. gasseri*, *L. paracasei* and *L. rhamnosus* were commonly detected from our subjects. The similarity of the faecal and salivary *Lactobacillus* populations found in this and another study (Dal Bello & Hertel, 2006) indicate that at least some *Lactobacillus* species/strains are able to live in different niches in the human orogastrointestinal tract, although the oral cavity and colon differ in several aspects including redox potential, nutrients, mucosal surfaces and co-existing members of the specific microbial community.

Between two and eight indigenous RAPD types were found per subject from faecal Beerens isolates and between two and nine per subject from salivary Beerens isolates. However, only one to seven faecal and zero to two salivary RAPD types per subject were confirmed to be bifidobacteria (after bifidobacteria-specific PCR with primers Bif164-f and Bif662-GC-r; Satokari *et al.*, 2001a) (Table 4), indicating that most salivary Beerens biotypes were not bifidobacteria. Whereas 80 % (37/46; Table 4) of the faecal Beerens biotypes were bifidobacteria. No identical indigenous RAPD types were found between saliva and faeces. After sequencing of the different bifidobacteria RAPD types, multiple indigenous bifidobacterial genotypes, including *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Bifidobacterium catenulatum/ Bifidobacterium pseudocatenulatum*, *Bifidobacterium bifidum* and *Bifidobacterium angulatum* were detected in faeces within an individual, whilst only *Bifidobacterium dentium* and *B. bifidum* were detected from saliva (Table 4). We found *B. dentium* in the saliva of five subjects. *B. dentium* was not found in faecal samples, which is consistent with a published culture-based analysis (Mättö *et al.*, 2004). However, it has been found in faeces in DNA-based studies (Matsuki *et al.*, 1999). We used culture-based identification in the present study, as we wanted to see whether the bifidobacteria detected from the faeces were alive and therefore most likely to represent autochthonous strains. The levels of *B. dentium* in our salivary samples gave a mean value of 2×10^5 c.f.u. ml^{-1}, which accounts for 10^6–10^8 cells in 1000 ml saliva; this is the mean quantity of saliva ingested daily (Nisengard & Newman, 1994). Therefore, the *B. dentium* strains found in the faecal samples by DNA-based methods may originate from saliva and may actually be allochthonous.

Conclusions

In conclusion, the predominant bacteria, bifidobacteria and Erec group bacteria, of the oral cavity and intestines were generally stable during probiotic consumption,

Table 3. Heterogeneity of indigenous *Lactobacillus* populations

Subject	Saliva		Faeces		Comparison of saliva and faeces	
	No. of RAPD types	Species*	No. of RAPD types	Species*	Identical RAPD types	Species*
1	5	*L. rhamnosus*, *L. casei*/*L. paracasei*, *L. brevis*	5	*L. brevis*	0	–
2	4	*L. rhamnosus*, *L. gasseri*, *L. paracasei*	4	*L. rhamnosus*, *L. gasseri*, *L. sakei*, *L. parabuchneri*	2	*L. rhamnosus*, *L. gasseri*
3	4	*L. rhamnosus*, *L. gasseri*, *L. brevis*, *L. plantarum* group†	1	*L. gasseri*	1	*L. gasseri*
4	6	*L. rhamnosus*, *L. paracasei*, *L. parabuchneri*, *L. plantarum* group†	2	*L. rhamnosus*, *L. parabuchneri*/*L. ferintoshensis*	1	*L. rhamnosus*
5	5	*L. rhamnosus*, *L. paracasei*, *L. jensenii*	3	*L. rhamnosus*, *L. paracasei*	1	*L. paracasei*
6	5	*L. gasseri*, *L. paracasei*, *L. parabuchneri*, *L. fermentum*	4	*L. gasseri*, *L. paracasei*, *L. rhamnosus*	1	*L. gasseri*
7	5	*L. rhamnosus*, *L. paracasei*, *L. vaginalis*, *L. plantarum* group†	4	*L. rhamnosus*, *L. crispatus*, *L. plantarum* group†	2	*L. rhamnosus*, *L. plantarum* group†
8	6	*L. gasseri*, *L. paracasei*, *L. salivarius*, *L. fermentum*, *L. kitasonis*/*L. crispatus*	1	*L. gasseri*	0	–
9	3	*L. rhamnosus*, *L. brevis*, *Lactobacillus* sp.	4	*L. paracasei*, *L. plantarum* group†, *Lactobacillus* sp.	1	*Lactobacillus* sp.
10	3	*L. rhamnosus*, *L. gasseri*, *L. casei*/*L. paracasei*	4	*L. rhamnosus*, *L. gasseri*, *L. barabucneri*	1	*L. gasseri*

*Determined by 16S rRNA gene sequencing.
†*L. plantarum* group: *L. plantarum*, *L. arizonensis* and *L. pentosus*.

Table 4. Heterogeneity of indigenous *Bifidobacterium* populations

Subject	Saliva			Faeces		
	No. of initial RAPD types*	No. of confirmed RAPD types†	Species‡	No. of initial RAPD types*	No. of confirmed RAPD types†	Species‡
1	5	2	*B. dentium*, *B. bifidum*	4	1	*B. longum*
2	6	2	*B. dentium*, *B. bifidum*	8	5	*B. longum*, *B. adolescentis*, *B. ruminantium/B. adolescentis*
3	4	2	*B. dentium*	3	2	*B. longum*
4	9	1	*B. dentium*	2	2	*B. longum*, *B. catenulatum/B. pseudocatenulatum*
5	3	0	–	4	4	*B. longum*, *B. adolescentis*
6	4	0	–	5	5	*B. longum*, *B. adolescentis*, *B. bifidum*, *B. angulatum*
7	6	0	–	8	7	*B. longum*, *B. adolescentis*, *B. bifidum*
8	6	0	–	4	4	*B. longum*, *B. adolescentis*, *B. catenulatum/B. pseudocatenulatum*
9	5	0	–	4	4	*B. longum*, *B. adolescentis*, *B. catenulatum/B. pseudocatenulatum*, *B. ruminantium/B. adolescentis*
10	2	1	*B. dentium*	4	3	*B. longum*, *B. adolescentis*

**Bifidobacterium*-like isolates that were initially RAPD typed.
†Number of RAPD types of the bifidobacterial isolates that gave a positive result with a bifidobacteria-specific PCR (Satokari *et al.*, 2001a).
‡Determined by 16S rRNA gene sequencing.

showing more diversity in faeces than saliva, and having different species compositions for the two sampling sites. In contrast, the lactobacilli, which are known to inhabit several ecological niches, showed temporal instability in both faeces and saliva. Furthermore, faecal and salivary samples contained identical indigenous *Lactobacillus* genotypes (*L. rhamnosus*, *L. gasseri*, *L. paracasei*, *L. plantarum* group and a *Lactobacillus* sp.) in most subjects.

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ARTICLE V

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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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

FEMS MICROBIOLOGY ECOLOGY

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.

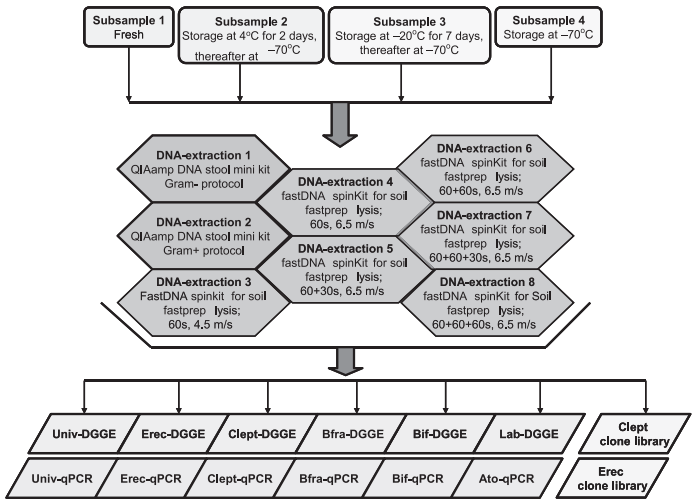

Fig. 1. The study plan. Univ, predominant bacteria; Erec, *Eubacterium rectale – Blautia coccoides* group; Clept, *Clostridium leptum* group; Bfra, *Bacteroides* spp. ; Lab, *Lactobacillus* group (comprises of genera *Lactobacillus, Leuconostoc, Pediococcus*, and *Weissella*); Ato, *Atopobium* group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

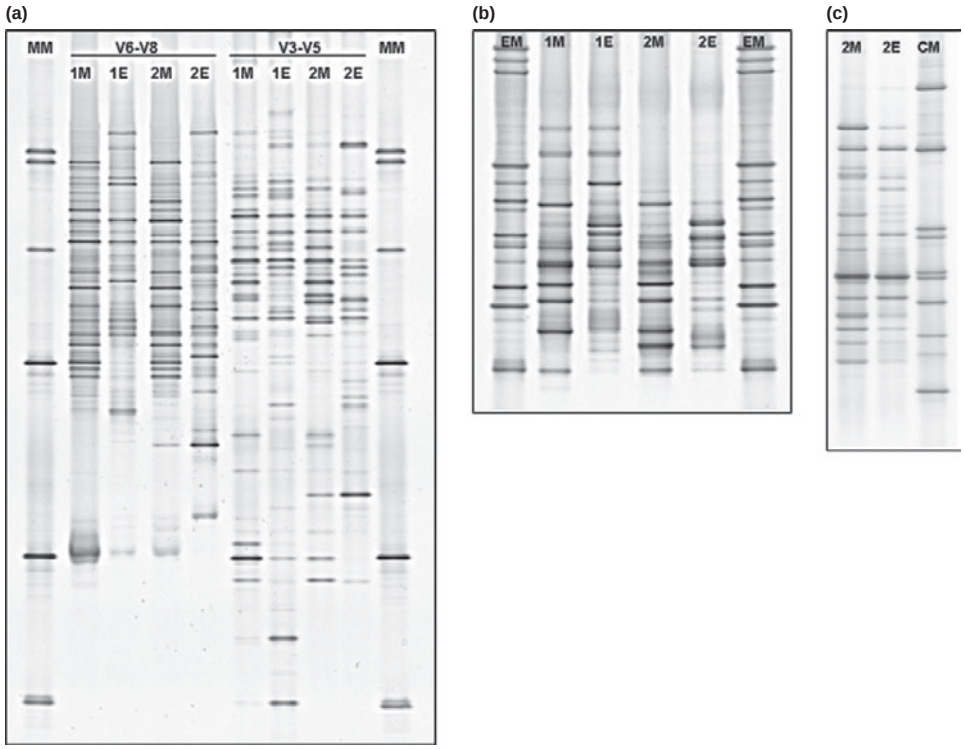

Fig. 2. (a) Predominant bacterial DGGE profiles as detected with amplification of 16S rRNA gene variable region V_6–V_8 (lanes 1–4) or V_3–V_5 (lanes 5–8); (b) *Eubacterium rectale* – *Blautia coccoides* group-specific DGGE profiles; and (c) *Clostridium leptum* group-specific DGGE profiles. 1M, sample 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.

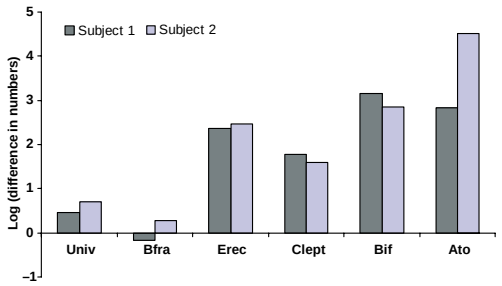

Fig. 3. Difference between numbers of bacterial groups obtained after different DNA-extraction protocols and qPCR from samples stored at −70 °C. The results are expressed as log-values (mechanical lysis – enzymatic lysis; i.e. positive number indicates more efficient mechanical lysis). All other differences, 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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Maukonen *et al.* FEMS Microbiol Ecol 2012, 79, 697-708.
Supplementary information

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Figure 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).

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Figure S6. Rarefaction curves of different clones per sample

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

Figure S8. Difference in numbers of *Bacteroides* spp. after all different storage combinations and mechanical DNA-extraction. The results are expressed as log-values (fresh – storage; i.e. positive numbers indicate higher numbers in fresh samples.

Supplementary references

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

Table S1.

Species	Strain	Clostridial cluster [a]	Phylogenetic affiliation according to NCBI taxonomy (Phylum, Family)
Alistipes finegoldii	VTT E-093113		Bacteroidetes, Rikenellaceae
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 dorei	DSM 17855		Bacteroidetes, Bacteroidaceae
Bacteroides eggerthii	VTT E-093118		Bacteroidetes, Bacteroidaceae
Bacteroides finegoldii	VTT E-093115		Bacteroidetes, Bacteroidaceae
Bacteroides fragilis	VTT E-022248		Bacteroidetes, Bacteroidaceae
Bacteroides intestinalis	VTT E-093114		Bacteroidetes, Bacteroidaceae
Bacteroides ovatus	VTT E-062944		Bacteroidetes, Bacteroidaceae
Bacteroides plebeius	DSM 17135		Bacteroidetes, Bacteroidaceae
Bacteroides thetaiotaomicron	VTT E-001738		Bacteroidetes, Bacteroidaceae
Bacteroides vulgatus	VTT E-001734		Bacteroidetes, Bacteroidaceae
Bifidobacterium adolescentis	VTT E-981074		Actinobacteria, Bifidobacteriaceae
Bifidobacterium angulatum	VTT E-001481		Actinobacteria, Bifidobacteriaceae
Bifidobacterium animalis subsp. *animalis*	VTT E-96663		Actinobacteria, Bifidobacteriaceae
Bifidobacterium animalis subsp. *lactis*	VTT E-97847		Actinobacteria, Bifidobacteriaceae
Bifidobacterium breve	VTT E-981075		Actinobacteria, Bifidobacteriaceae
Bifidobacterium catenulatum	VTT E-11764		Actinobacteria, Bifidobacteriaceae
Bifidobacterium longum subsp. *infantis*	VTT E-97796		Actinobacteria, Bifidobacteriaceae
Bifidobacterium longum subsp. *longum*	VTT E-96664		Actinobacteria, Bifidobacteriaceae
Blautia coccoides	VTT E-052778	XIV	Firmicutes, Incertae Sedis XIV

Blautia producta	VTT E-052786	XIV	Firmicutes, Incertae Sedis XIV
Clostridium acetobutylicum	VTT E-93498	I	Firmicutes, Clostridiaceae
Clostridium beijerinckii	VTT E-93498	I	Firmicutes, Clostridiaceae
Clostridium bolteae	VTT E-052776	XIV	Firmicutes, unclassified_Clostridiales (phylogenetically Lachnospiraceae)
Clostridium butyricum	VTT E-97426	I	Firmicutes, Clostridiaceae
Clostridium clostridioforme	VTT E-052777	XIV	Firmicutes, unclassified_Clostridiales (phylogenetically Lachnospiraceae)
Clostridium hathawayi	VTT E-062951	XIV	Firmicutes, unclassified_Clostridiales (phylogenetically 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 lituseburense	VTT E-021853	XI	Firmicutes, Peptostreptococcaceae
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
Desulfobacter curvatus	VTT E-001657		Proteobacteria, Desulfobacteraceae
Desulfobacterium autotrophicum	VTT E-001658		Proteobacteria, Desulfobacteraceae
Desulfosarcina variabilis	VTT E-001656		Proteobacteria, Desulfobacteraceae
Desulfotomaculum nigrificans	VTT E-001654		Firmicutes, Peptococcaceae
Desulfovibrio desulfuricans subsp. *desulfuricans*	VTT E-95573		Proteobacteria, Desulfovibrionaceae
Desulfovibrio piger	DSM 749		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
Fusobacterium nucleatum subsp. *nucleatum*	VTT E-052770		Fusobacteria, Fusobacteriaceae
Klebsiella terrigena	VTT E-96696		Proteobacteria, Enterobacteriaceae
Lachnospira multipara	VTT E-052784	XIV	Firmicutes, Lachnospiraceae
Lactobacillus brevis	VTT E-91458		Firmicutes, Lactobacillaceae
Lactobacillus casei	VTT E-85225		Firmicutes, Lactobacillaceae
Lactobacillus paracasei	VTT E-93490		Firmicutes, Lactobacillaceae
Lactobacillus plantarum	VTT E-79098		Firmicutes, Lactobacillaceae
Lactobacillus rhamnosus	VTT E-97800		Firmicutes, Lactobacillaceae
Lactobacillus salivarius	VTT E-97853		Firmicutes, Lactobacillaceae
Megasphaera elsdenii	VTT E-84221	IX	Firmicutes, Veillonellaceae
Methanobrevibacter smithii	DSM 861		Euryarchaeota, Methanobacteriaceae
Methanosphaera stadtmanae	DSM 3091		Euryarchaeota, Methanobacteriaceae
Parabacteroides distasonis	VTT E-062943		Bacteroidetes, Porphyromonadaceae
Parabacteroides merdae	VTT E-062953		Bacteroidetes, Porphyromonadaceae
Parascardovia denticolens	VTT E-991434		Actinobacteria, Bifidobacteriaceae
Pectinatus cerevisiiphilis	VTT E-79103	IX	Firmicutes, Veillonellaceae
Pediococcus acidilactici	VTT E-93493		Firmicutes, Lactobacillaceae
Prevotella intermedia	DSM 20706		Bacteroidetes, Prevotellaceae
Prevotella melaninogenica	VTT E-052771		Bacteroidetes, Prevotellaceae
Roseburia intestinalis	VTT E-052785	XIV	Firmicutes, Lachnospiraceae
Ruminococcus obeum	VTT E-052772	XIV	Firmicutes, Incertae Sedis XIV
Scardovia inopinatum	VTT E-991435		Actinobacteria, Bifidobacteriaceae
Selenomonas lacticifex	VTT E-90407	IX	Firmicutes, Veillonellaceae
Subdoligranulum variabile	VTT E-062950	IV	Firmicutes, Ruminococcaceae
Veillonella parvula	VTT E-001737	IX	Firmicutes, Veillonellaceae

[a] Clostridial phylogenetic cluster number (Collins et al., 1994).

Table S2. Primers used in the present study.

Target group	Short name	Probe / primer	Use	Sequence (5' → 3')	Reference or Source
predominant bacteria [a]	Univ	U968-f +GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACG CGAAGAACCTTA	(Nübel et al., 1996)
predominant bacteria [a]	Univ	U1401-r	PCR-DGGE	CGGTGTGTACAAGACCC	(Nübel et al., 1996)
predominant bacteria [b]		358F + GC	PCR-DGGE	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTA CGGGAGGCAGCAG	(Muyzer et al., 1993)
predominant bacteria [b]		534R	PCR-DGGE qPCR	ATTACCGCGGCTGCTGG	(Muyzer et al., 1993)
predominant bacteria [b]		358F	qPCR	CCT ACG GGA GGC AGC AG	(Muyzer et al., 1993)
Eubacterium rectale – Blautia coccoides group [c]	Erec-group	Ccoc-f	PCR-DGGE	AAATGACGGTACCTGACTAA	(Matsuki et al., 2002)
Eubacterium rectale – Blautia coccoides group [c]	Erec-group	Ccoc-r + GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGCTTT GAGTTTCATTCTTGCGAA	(Maukonen et al., 2006)
Eubacterium rectale – Blautia coccoides group [c]	Erec-group	g-Ccoc-F	qPCR	AAATGACGGTACCTGACTAA	(Matsuki et al., 2004)
Eubacterium rectale – Blautia coccoides group [c]	Erec-group	g-Ccoc-R	qPCR	CTTTGAGTTTCATTCTTGCGAA	(Matsuki et al., 2004)
Clostridium leptum group [d]	Clept-group	Clept-933 f	PCR-DGGE	GCACAAGCAGTGGAGT	(Matsuki et al., 2004)
Clostridium leptum group [d]	Clept-group	Clept-1240-r		GTT TTR TCA ACG GCA GTC	(Sghir et al., 2000)
Clostridium leptum group [d]	Clept-group	Clept-1240-r+GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGGTTT TRTCAACGGCAGTC	this study
Clostridium leptum group [d]	Clept-group	Clept-f	qPCR	GCACAAGCAGTCGAGT	(Matsuki et al., 2004)
Clostridium leptum group [d]	Clept-group	Clept-R3	qPCR	CTTCCTCCGTTTTGTCAA	(Matsuki et al., 2004)
genus *Bacteroides*	*Bacteroides* spp.	Bact596f	PCR-DGGE	TCA GTT GTG AAA GTT TGCG	(Vanhoutte et al., 2004)
		Bacto1080r		GCA CTT AAG CCG ACA CCT	(Doré et al., 1998)
genus *Bacteroides*	*Bacteroides* spp.	Bacto1080r + GC	PCR-DGGE	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGGCAC TTAAGCCGACACCT	this study

genus *Bacteroides*	*Bacteroides* spp	g-Bfra-F	qPCR	ATAGCCTTTCGAAAGRAAGAT	(Matsuki et al., 2004)
genus *Bacteroides*	*Bacteroides* spp	g-Bfra-R	qPCR	CCAGTATCAACTGCAATTTTA	(Matsuki et al., 2004)
Bifidobacteria	Bif	Bif164-f	PCR-DGGE	GGGTGGTAATGCCGGATG	(Satokari et al., 2001)
Bifidobacteria	Bif	Bif662-GC-r	PCR-DGGE	CGCCCGCCGCGCGCGGCGGGCCGGGCGGGGGCACGGGGGGCCAC CGTTAGACCGGGAA	(Satokari et al., 2001)
Bifidobacteria	Bif	Bifid-f	qPCR	CTCCTGGAAACGGGTGG	(Matsuki et al., 2002)
Bifidobacteria	Bif	Bifid-r	qPCR	GGTGTTCTTCCCGATATCTACA	(Matsuki et al., 2002)
Lactobacillus-group[e]	Lac	Lac1	PCR-DGGE	AGCAGTAGGGAATCTTCCA	(Vanhoutte et al., 2004)
Lactobacillus-group[e]	Lac	Lac2GC	PCR-DGGE	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCG CCCCCGCCCCATTYCACCGCTACACATG	(Vanhoutte et al., 2004)
Atopobium-group[f]	Ato	Atopo-f	qPCR	GGGTTGAGAGACCGACC	(Matsuki et al., 2004)
Atopobium-group[f]	Ato	Atopo-r	qPCR	CGGRGCTTCTTCTGCAGG	(Matsuki et al., 2004)
sequencing		T7	seq.	TAATACGACTCACTATAGG	Promega
sequencing		SP6	seq.	GATTTAGGTGACACTATAG	Promega

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

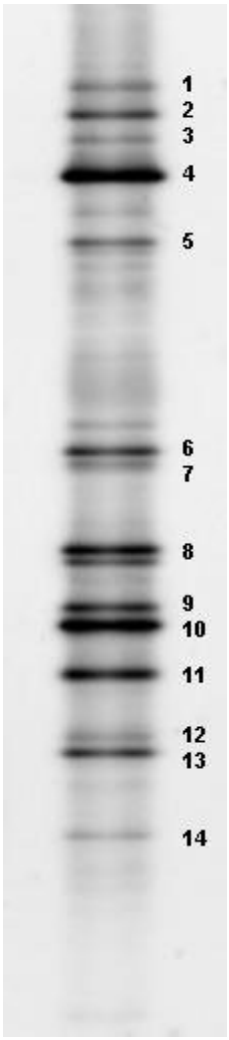
[b] Partial 16S rRNA gene (V_3-V_5 hypervariable region)

[c] Clostridial phylogenetic clusters XIVa (Collins et al., 1994)

[d] Clostridial phylogenetic clusters IV (Collins et al., 1994)

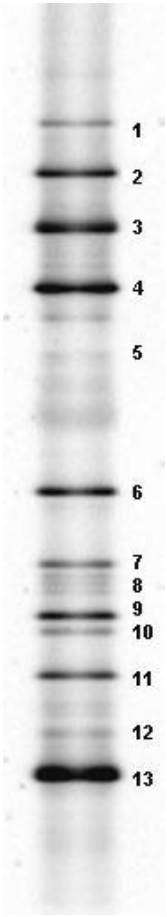
[e] *Lactobacillus*-group comprises of the genera *Lactobacillus, Leuconostoc, Pediococcus*, and *Weissella*

[f] *Atopobium*-group comprises e.g. of genera *Atopobium*, *Eggerthella*, and *Collinsella*



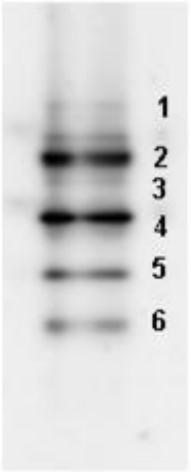
Band number	Blast results	similarity	clones
1	*Ruminococcus callidus*, type strain ATCC 27760	93 %	1
2	*Faecalibacterium prausnitzii*, type strain ATCC 27768	94 %	2
3	*Ruminococcus bromii* , type strain ATCC 27255	96 %	1
4	*Ruminococcus bromii* , type strain ATCC 27255	97-99%	42
	Ruminococcus albus , type strain ATCC 27210	95-96%	3
	Clostridium methylpentosum , type strain DSM 5476	95-97%	2
5	*Faecalibacterium prausnitzii* , type strain ATCC 27768	93 %	1
6	*Subdoligranulum variabile* , type strain BI 114T	96 %	4
7	*Faecalibacterium prausnitzii* , type strain ATCC 27768	92 %	2
8	*Faecalibacterium prausnitzii* , type strain ATCC 27768	99-100%	5
9	*Faecalibacterium prausnitzii* , type strain ATCC 27768	92 %	3
10	*Subdoligranulum variabile* , type strain BI 114T	99-100%	16
	Eubacterium siraeum , type strain ATCC 29066	96 %	3
11	*Clostridium sporosphaeroides* , type strain ATCC 25781	97 %	2
12	*Eubacterium desmolans* , type strain ATCC 43058	93 %	1
13	*Faecalibacterium prausnitzii* , type strain ATCC 27768	94-95%	8
14	*Faecalibacterium prausnitzii* , type strain ATCC 27768	95-96%	4
n.d.	*Eubacterium desmolans* , type strain ATCC 43058	91 %	1
n.d.	*Eubacterium plautii* , type strain CCUG 28093	89 %	1
n.d.	*Faecalibacterium prausnitzii* , strain ATCC 27768	94 %	1
n.d.	*Faecalibacterium prausnitzii* , strain ATCC 27768	93 %	1
n.d.	*Faecalibacterium prausnitzii* , strain ATCC 27768	93 %	1
n.d.	*Faecalibacterium prausnitzii* , strain ATCC 27768	93 %	1
n.d.	*Faecalibacterium prausnitzii* , strain ATCC 27768	93 %	1
n.d.	*Faecalibacterium prausnitzii* , strain ATCC 27768	93 %	1
n.d.	*Ruminococcus bromii* , type strain ATCC 27255	98 %	1
n.d.	*Ruminococcus callidus* , type strain ATCC 27760	92 %	1
n.d.	*Ruminococcus flavefaciens* , type strain ATCC 19208	99 %	1
n.d.	*Ruminococcus flavefaciens* , type strain ATCC 19208	94 %	1
n.d.	*Subdoligranulum variabile* , type strain BI 114T	96 %	1
n.d.	*Subdoligranulum variabile* , type strain BI 114T	94 %	1
n.d.	*Clostridium aldrichii* , type strain DSM 6159	91 %	1

Figure 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).



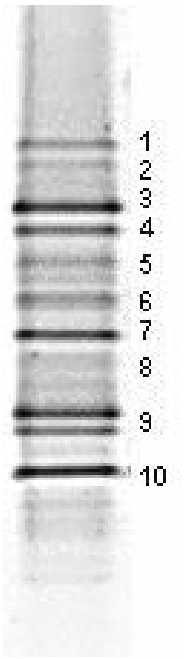
Band number	Blast results	similarity	clones
1	*Faecalibacterium prausnitzii*, type strain ATCC 27768	94 %	1
2	*Faecalibacterium prausnitzii*, type strain ATCC 27768	93 %	9
3	*Faecalibacterium prausnitzii*, type strain ATCC 27768	94 %	3
	Ruminococcus albus, type strain ATCC 27210	96 %	9
4	*Faecalibacterium prausnitzii*, type strain ATCC 27768	92-93%	13
5	*Faecalibacterium prausnitzii*, type strain ATCC 27768	93 %	2
6	*Faecalibacterium prausnitzii*, type strain ATCC 27768	91-92%	5
7	*Faecalibacterium prausnitzii*, type strain ATCC 27768	99-100%	2
8	*Clostridium alkalicellulosi*, type strain Z-7026	90 %	2
9	*Faecalibacterium prausnitzii*, type strain ATCC 27768	91-92%	6
10	*Subdoligranulum variabile*, type strain BI 114T	100 %	1
11	*Faecalibacterium prausnitzii*, type strain ATCC 27768	93 %	4
12	*Eubacterium desmolans*, type strain ATCC 43058	93 %	2
13	*Subdoligranulum variabile*, type strain BI 114T	97-98%	22
n.d.	*Faecalibacterium prausnitzii*, type strain ATCC 27768	94 %	1
n.d.	*Faecalibacterium prausnitzii*, type strain ATCC 27768	93 %	1
n.d.	*Faecalibacterium prausnitzii*, type strain ATCC 27768	92 %	1
n.d.	*Ruminococcus albus*, type strain ATCC 27210	90 %	1

Figure 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).



Band number	BLAST results	similarity	clones
1	*Bacteroides fragilis*, type strain NCTC 9343	100 %	1
2	*Bacteroides vulgatus* type strain ATCC 8482	97-100%	43
3	*Bacteroides ovatus*, type strain JCM5824	99 %	1
4	*Bacteroides uniformis*, type strain JCM5828	97-100%	57
5	*Bacteroides thetaiotaomicron*, type strain VPI-5482	97 %	4
6	*Bacteroides dorei*, type strain JCM 13471	99-100%	5
n.d.	*Bacteroides acidifaciens*, type strain JCM10556	99 %	1
n.d.	*Bacteroides caccae*, type strain JCM9498	100 %	1
n.d.	*Bacteroides dorei*, type strain:JCM 13471	97 %	1
n.d.	*Bacteroides uniformis*, type strain JCM5828	98 %	1
n.d.	*Bacteroides vulgatus*, type strain ATCC 8482	97 %	1
n.d.	*Bacteroides vulgatus*, type strain ATCC 8482	99 %	1
n.d.	*Bacteroides vulgatus*, type strain ATCC 8482	99 %	1
n.d.	*Bacteroides vulgatus*, type strain ATCC 8482	99 %	1

Figure 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).



Band number	Blast results	similarity	clones
1	*Bacteroides fragilis*, type strain NCTC 9343	99-100%	4
1 + 2	*Bacteroides ovatus*, type strain CIP 103756	98 %	2
	Bacteroides caccae, type strain JCM9498	98 %	1
3	*Bacteroides ovatus*, type strain CIP 103756	99 %	7
3 + 4	*Bacteroides vulgatus*, type strain ATCC 8482	99 %	25
	Bacteroides dorei, type strain JCM 13471	99 %	3
4	*Bacteroides xylanisolvens*, type strain XB1AT	96 %	1
5 (1 band)	*Bacteroides ovatus*, type strain CIP 103756	97 %	4
5 (2 bands)	*Bacteroides thetaiotaomicron*, type strain VPI-5482	99 %	5
6	*Bacteroides thetaiotaomicron*, type strain VPI-5482	99-100%	6
	Bacteroides caccae, type strain JCM9498	97 %	1
	Bacteroides eggerthii, type strain JCM12986	98 %	1
7	*Bacteroides eggerthii,* type strain JCM12986	98-99%	16
8	*Bacteroides dorei*, type strain JCM 13471	96 %	1
9 (double band)	*Bacteroides dorei*, type strain JCM 13471	99-100%	21
	Bacteroides vulgatus, type strain ATCC 8482	98-99%	2
10	*Bacteroides eggerthii,* type strain JCM12986	97-99%	16
n.d.	*Bacteroides dorei*, type strain JCM 13471	98 %	1
n.d.	*Bacteroides dorei*, type strain JCM 13471	99 %	1
n.d.	*Bacteroides dorei*, type strain JCM 13471	98 %	1
n.d.	*Bacteroides ovatus*, type strain CIP 103756	96 %	1
n.d.	*Bacteroides thetaiotaomicron*, type strain VPI-5482	96 %	1

Figure 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).

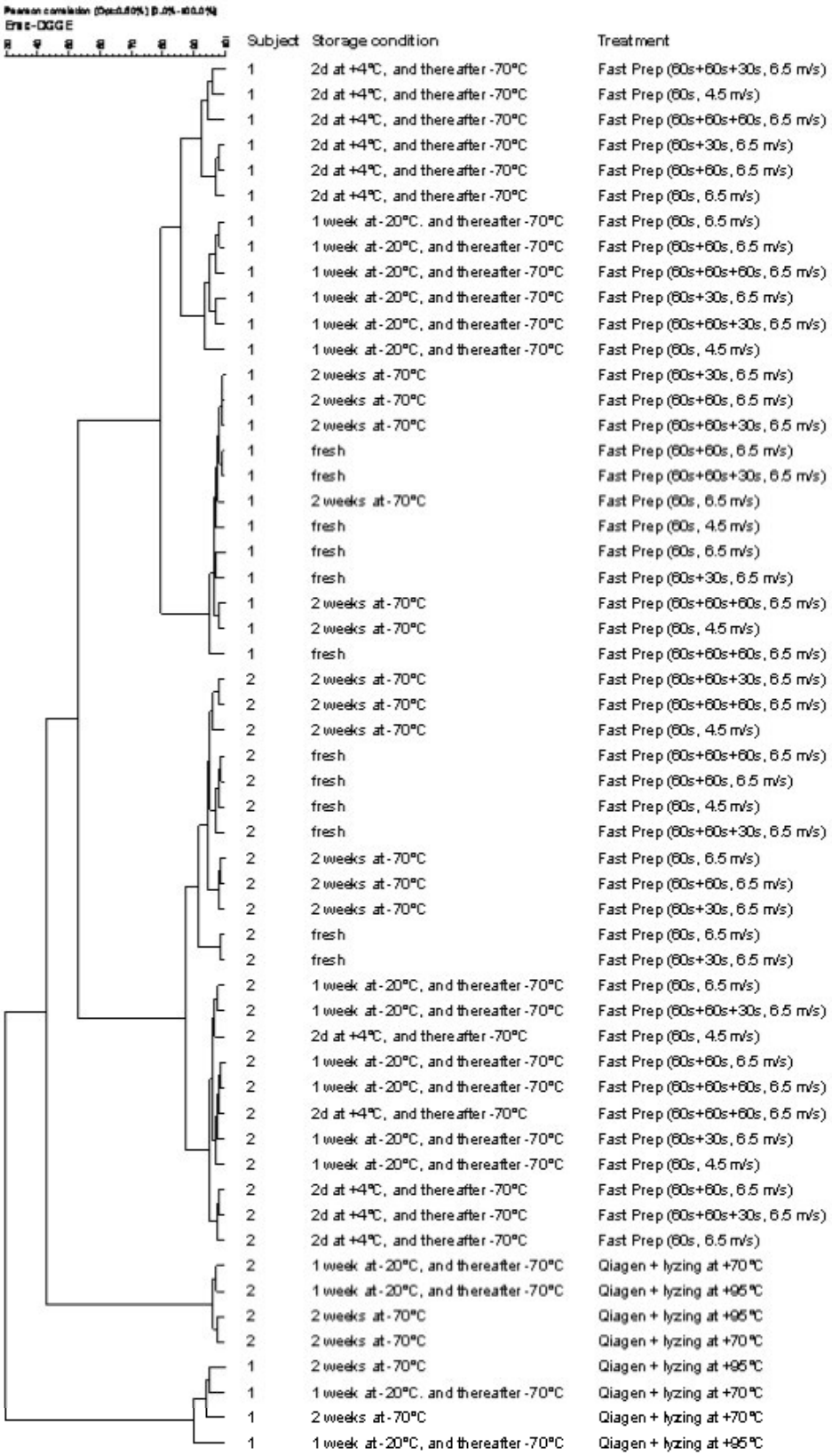


Figure S5. Clustering of Erec-group DGGE-profiles of both subjects after different storage – DNA-extraction combinations. Only one of the replicate samples was included in the presented clustering.

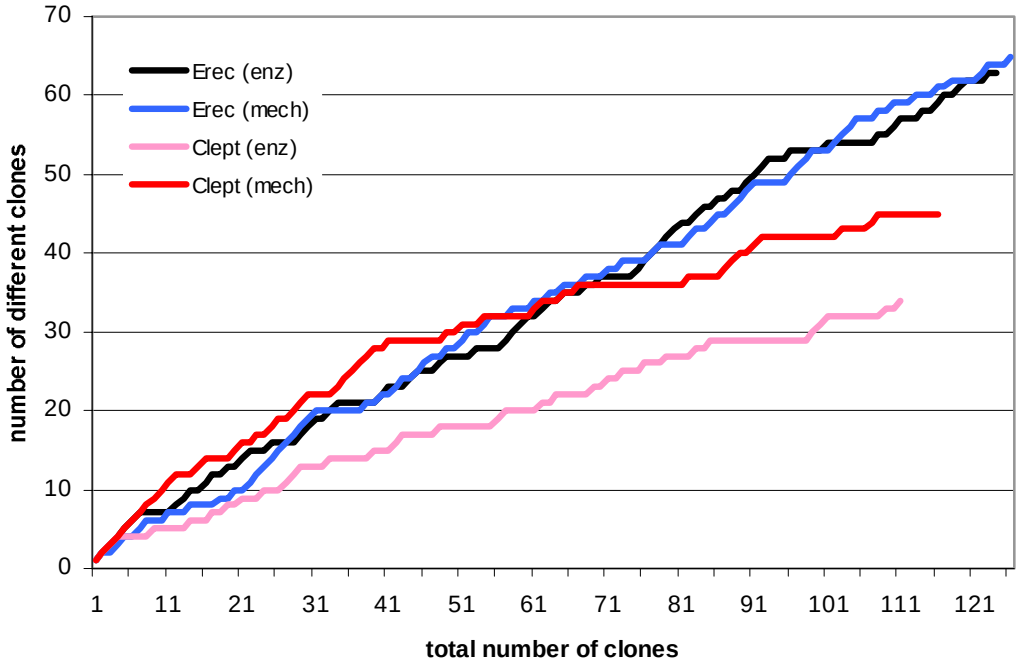


Figure S6. Rarefaction curves of different clones per sample

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

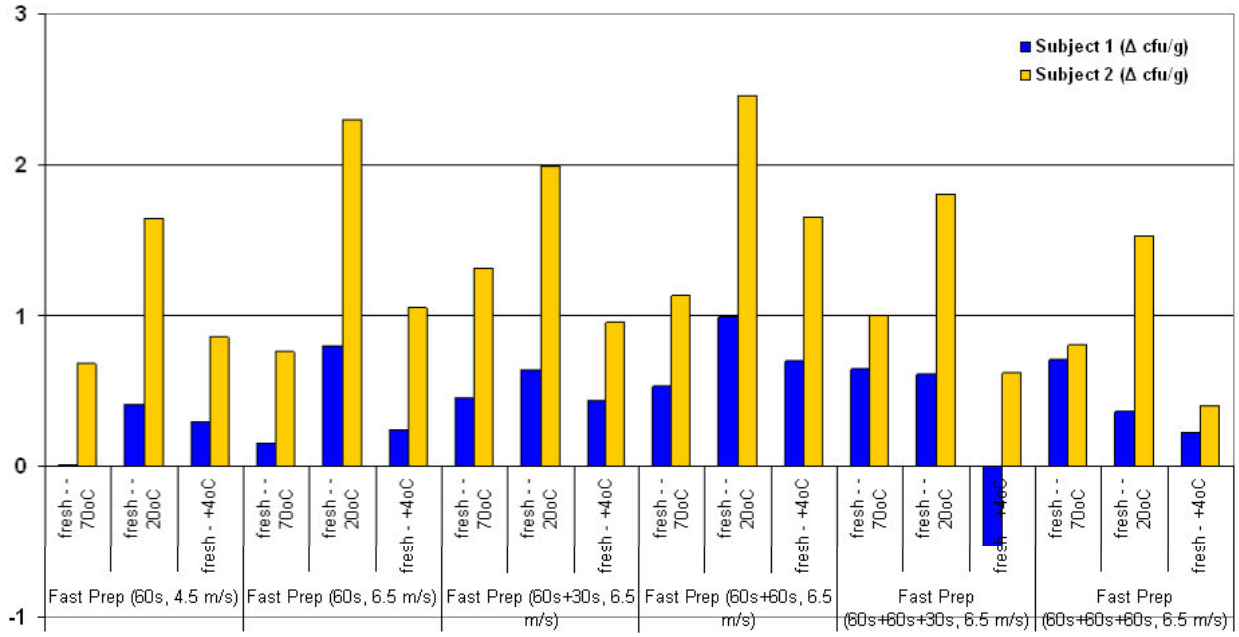


Figure S8. Difference in numbers of *Bacteroides* spp. after all different storage combinations and mechanical DNA-extraction. The results are expressed as log-values (fresh – storage; i.e. positive numbers indicate higher numbers in fresh samples.

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Series title and number
VTT Science 26

Title	**Characterization of the human predominant fecal microbiota** **With special focus on the Clostridial clusters IV and XIVa**
Author(s)	Johanna Maukonen
Abstract	The human gut microbiota is considered to be a complex fermentor with a metabolic potential rivaling that of the liver. In addition to its primary function in digestion, the indigenous microbial community has an important influence on host physiological, nutritional and immunological processes. The primary aim of this study was to characterize human predominant fecal microbiota with a special focus on Clostridial clusters XIV (Lachnospiraceae, *Eubacterium rectale – Blautia coccoides* group) and IV (Ruminococcaceae, *Clostridium leptum* group). The specific aims were: 1) To develop molecular methods for characterization of the human predominant fecal microbiota; 2) To assess the specificity, practicality, and usability of the developed methods for human fecal samples in healthy adults, elderly people, and people having IBS; 3) To assess possible confounding factors in the analysis of human fecal samples. Molecular tools were developed for sensitive and highly specific characterization of the human predominant fecal and salivary microbiota. DNA- and rRNA-based denaturing gradient gel electrophoresis methods (DGGE) were developed for *Eubacterium rectale – Blautia coccoides* group (Erec), rRNA-based DGGE method for predominant bacteria, and DNA-based DGGE methods for *Clostridium leptum* group (Clept) and *Bacteroides* spp. In addition, quantitative real-time PCR (qPCR) methods targeting predominant bacteria, Erec-group, Clept-group, *Bacteroides* spp., bifidobacteria, and *Atopobium* group were developed. Predominant bacterial, Erec-group, Clept-group, and *Bacteroides* spp. populations of healthy adults were temporally rather stable, showing intra-individual diversity and inter-individual variability. The rRNA-based profiles showed more temporal instability than DNA-based profiles. The enumerated clostridial groups (Erec, Clept, *C. lituseburense*, and *C. histolyticum*) represented the dominant fecal microbiota of most of the studied subjects, comprising altogether 29–87% of the total bacteria. Erec-group was the dominant group, accounting on average for 43% of total bacteria in control subjects, and 30% and 50% in irritable bowel syndrome (IBS) subjects affected by constipation and diarrhea, respectively. The observations indicated that in addition to temporal instability of the active predominant fecal bacterial population, clostridial microbiota may be involved in IBS. Differences in the predominant fecal microbiota between elderly people and younger adults were also assessed. Temporal stabilities of the studied bacterial populations (predominant bacteria, Erec-group, bifidobacteria, and lactobacilli) were similar in both age groups. However, the diversity of predominant bacteria and Erec-group bacteria was significantly higher in elderly subjects as compared to younger adults. Consumption of probiotic yoghurt containing galacto-oligosaccharide (GOS) for three weeks did not significantly affect the diversity or temporal stability of the studied bacterial groups. However, the composite data set containing all DGGE analyses of the study showed that the microbial communities from the GOS-supplemented subjects were more similar to each other than those of the control subjects. The similarity of the salivary and fecal microbiota was studied to assess whether the upper gastrointestinal tract microbiota influence the results obtained with DNA-based methods from feces. The predominant bacteria, bifidobacteria, and Erec-group bacteria of the oral cavity and feces were generally stable during probiotic consumption, showing more diversity in feces than in saliva and different species composi-tions for the two sampling sites. Lactobacilli, however, showed temporal instability in both feces and saliva. Furthermore, fecal and salivary samples contained identical indigenous *Lactobacillus* genotypes in most subjects. The effects of storage conditions and DNA-extraction protocols of fecal samples on the results were also evaluated. The DNA-extraction did not affect the diversity, composition, or quantity of *Bacteroides* spp., whereas after one week's storage at -20^{0}C the numbers of *Bacteroides* spp. were 1.6–2.5 log-units lower. Furthermore, the numbers of predominant bacteria, Erec-group, Clept-group, bifidobacteria, and *Atopobium*-group were 0.5–4 log-units higher after mechanical DNA-extraction than after enzymatic DNA-extraction as detected with qPCR, regardless of the storage. The bacterial composition of Erec-group differed significantly depending on the DNA-extraction protocol;. According to the results, rigorous mechanical lysis leads to the detection of higher bacterial numbers and diversity from human fecal samples than enzymatic DNA-extraction. Since 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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Julkaisun sarja ja numero
VTT Science 26

Nimeke	**Ihmisen vallitsevan ulostemikrobiston karakterisointi** **Painopiste erityisesti klostridien fylogeneettisissä ryhmissä IV ja XIVa**
Tekijä(t)	Johanna Maukonen
Tiivistelmä	Suolistomikrobiston ensisijainen tehtävä on auttaa ruoansulatuksessa; paksusuolen mikrobit käyttävät hyödykseen useita ravintoaineita, jotka eivät imeydy ohutsuolessa. Ihminen puolestaan hyödyntää mikrobien aineenvaihduntatuotteita. Suolistomikrobisto vaikuttaa ihmiseen monella muullakin tavoin: se edesauttaa immuunijärjestelmän kehittymistä, tuottaa kaasuja sekä lyhytketjuisia rasvahappoja, muuttaa sappihappojen muotoa, tuottaa vitamiineja, ja muodostaa mahdollisesti myös mutageenisiä, toksisia sekä karsinogeenisiä yhdisteitä. Niinpä ihmisen suolistomikrobistolla on merkittävä vaikutus ihmisen fysiologisiin, ravitsemuksellisiin ja immunologisiin toimintoihin. Tämän tutkimuksen tavoitteena oli kehittää menetelmiä ihmisen suolistomikrobiston tärkeimpien bakteeriryhmien karakterisointiin ja kvantitointiin, sekä todentaa kehitettyjen menetelmien soveltuvuus ihmisen uloste- ja sylkinäytteiden analysointiin. Tässä työssä kehitettiin spesifiset ja herkät rRNA-pohjaiset PCR – denaturoiva gradientti geeli elektroforeesi (DGGE) -menetelmät vallitsevan bakteeriston ja *Eubacterium rectale – Blautia coccoides* -klostridiryhmän (Erec) karakterisointiin sekä DNA-pohjaiset PCR-DGGE menetelmät *Clostridium leptum* -klostridiryhmän (Clept), sekä *Bacteroides*-suvun karakterisointiin. Lisäksi kehitettiin reaaliaikaiset PCR-menetelmät (qPCR) vallitsevan bakteeriston, Erec-ryhmän, Clept-ryhmän, *Bacteroides*-suvun, bifidobakteerien ja *Atopobium*-ryhmän kvantitointiin. Terveiden aikuisten vallitseva bakteeriston, Erec-klostridiryhmän, Clept-klostridiryhmän sekä *Bacteroides*-suvun bakteeripopulaatiot olivat ajallisesti melko stabiileja. Bakteeripopulaatioprofiilit olivat monimuotoisia ja kullekin ihmiselle ominaisia. rRNA:han pohjautuvat bakteeriprofiilit olivat sekä terveillä aikuisilla että ärtyneen suolen oireyhtymästä (IBS) kärsivillä aikuisilla ajallisesti epästabiilimpia kuin DNA-pohjaiset bakteeriprofiilit. Lisäksi IBS-potilaiden rRNA-pohjaiset vallitsevan bakteeriston profiilit olivat ajallisesti epästabiilimpia kuin terveiden aikuisten. Tutkittujen klostridiryhmien (Erec, Clept, *C. lituseburense* –ryhmä sekä *C. histolyticum* –ryhmä) bakteerit muodostivat valtaosan (29–87 %) ulostemikrobistosta. Erec-ryhmän klostridit muodostivat keskimäärin 43 % terveiden aikuisten ulostemikrobistosta, kun taas IBS-potilailla Erec-ryhmän klostridit muodostivat 30 % (ummetustyyppi) tai 50 % (ripulityyppi) ulostemikrobistosta. Aikuisten ja vanhempien ihmisten (yli 60 v.) välillä ei ollut eroja tutkittujen bakteeriryhmien ajallisessa stabiilisuudessa (vallitseva bakteeristo, Erec- ryhmän klostridit, bifidobakteerit ja laktobasillit). Vanhempien ihmisten vallitseva bakteeristo sekä Erec-klostridiryhmä olivat kuitenkin monimuotoisempia kuin nuorempien aikuisten. Koska DNA-pohjaisilla menetelmillä voitaisiin periaatteessa detektoida ulosteesta myös sylkibakteereita, tutkimuksesssa selvitettiin myös löytyykö syljestä samoja bakteereita kuin ulosteesta. Sekä syljen että ulosteen vallitseva bakteeristo, Erec-ryhmän klostridit, bifidobakteeri- ja laktobasillipopulaatiot pysyivät melko stabiileina probiootti-syöttöjakson aikana. Ulostenäytteiden bakteeripopulaatiot olivat monimuotoisempia kuin syljen bakteeripopulaatiot. Lisäksi näiden bakteerisuvut/lajit olivat pääasiallisesti erilaisia. Ainoan selkeän poikkeuksen muodostivat laktobasillit; useimpien koehenkilöiden syljestä ja ulosteesta löytyi samoja laktobasilli-lajeja ja toisinaan jopa kantoja. Viimeisessä osatyössä tutkittiin ulostenäytteiden säilytyslämpötilojen sekä eri DNA-eristysmenetelmien vaikutusta näytteistä saataviin tuloksiin. Näytteiden säilytys -20°C:ssa viikon ajan alensi *Bacteroides*-suvun bakteerien lukumäärää 1,6–2,5 log-yksikön verran, kun taas eri DNA-eristysmenetelmillä ei ollut vaikutusta *Bacteroides*-suvun bakteerien määrään tai populaation monimuotoisuuteen tai koostumukseen. Säilytyslämpötila ei vaikuttanut merkitsevästi muiden tutkittujen bakteeriryhmien määriin eikä myöskään populaatioiden monimuotoisuuteen tai koostumukseen. Käytetyllä DNA-eristysmenetelmällä sen sijaan oli merkittävä vaikutus: Vallitseva bakteeriston, Erec-ryhmän klostridien, Clept-ryhmän klostridien, bifidobakteerien ja *Atopobium*-ryhmän bakteerien määrät olivat 0,5–4 log-yksikköä korkeammat mekaanista DNA-eristystä käytettäessä kuin entsymaattista DNA-eristystä käytettäessä. Lisäksi käytetyllä DNA-eristysmenetelmällä oli merkittävä vaikutus Erec-ryhmän bakteerikoostumukseen. Käytetyllä DNA-eristysmenetelmällä on saatujen tulosten perusteella huomattava merkitys etenkin klostridi- sekä aktinobakteeripopulaatioden detektointiin ja kvantitointiin. Eri DNA-eristysmenetelmien käyttö saattaakin osittain selittää viimeaikoina saatuja ristiriitaisia tuloksia liittyen esim. suolistomikrobiston rooliin lihavuudessa, sekä bifidobakteerien määrään vauvojen suolistossa.
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Characterization of the human predominant fecal microbiota

With special focus on the Clostridial clusters IV and XIVa

The human gut microbiota is considered to be a complex fermentor with a metabolic potential rivaling that of the liver. In addition to its primary function in digestion, the indigenous microbial community has an important influence on host physiological, nutritional and immunological processes.

Molecular tools were developed for rapid, sensitive, and highly specific characterization of the human predominant fecal and salivary microbiota. Predominant bacterial, *Eubacterium rectale – Blautia coccoides group* (Erec), *Clostridium leptum* group (Clept), and *Bacteroides* spp. populations of healthy adults were temporally rather stable, showing intra-individual diversity and inter-individual variability. However, rRNA-based denaturing gradient gel electrophoresis (DGGE) profiles showed more temporal instability than DNA-based profiles. Moreover, the diversity of predominant bacteria and Erec-group bacteria was significantly higher in elderly subjects as compared to younger adults. Clostridial populations represented the dominant fecal microbiota of most of the studied subjects. However, the proportion of Erec-group was significantly lower in the constipation type IBS subjects than in the healthy adults. Altogether, the observations indicated that in addition to temporal instability of the active predominant fecal bacterial population, clostridial microbiota may be involved in IBS. Thereafter, the similarity of the salivary and fecal microbiota was studied to assess whether the upper gastrointestinal tract microbiota influence the results obtained with DNA-based methods from feces. The predominant bacteria, bifidobacteria, and Erec-group bacteria of the oral cavity and feces showed more diversity in feces than in saliva and different species compositions for the two sampling sites. However, fecal and salivary samples contained identical indigenous *Lactobacillus* genotypes in most subjects. The effects of storage conditions and DNA-extraction protocols of fecal samples on the results were also evaluated. The DNA-extraction did not affect the diversity, composition, or quantity of *Bacteroides* spp., whereas after one week's storage at -20°C the numbers of *Bacteroides* spp. were significantly lower. Furthermore, the numbers of predominant bacteria, Erec-group, Clept-group, bifidobacteria, and *Atopobium*-group were 0.5–4 log-units higher after mechanical DNA-extraction than after enzymatic DNA-extraction, regardless of the storage. Moreover, the bacterial composition of Erec-group differed significantly depending on the DNA-extraction protocol applied. According to the results, rigorous mechanical lysis leads to the detection of higher bacterial numbers and diversity from human fecal samples than enzymatic DNA-extraction.

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