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DIPLOMARBEIT

Titel der Diplomarbeit

Effects of ageing and vegetarianism on diversity and population sizes of *Clostridium* cluster IV assessed with TaqMan PCR and PCR-DGGE.

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Verfasserin:	Kathrin Ingrid Liszt
Matrikelnummer:	0306712
Studienrichtung:	Ernährungswissenschaften (A474)
Betreuer:	Alexander Haslberger

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The results of this diploma thesis have been published in two articles:

Kathrin Liszt, Jutta Zwielehner, Michael Handschur, Berit Hippe, Roman Thaler, Alexander G. Haslberger; Characterization of *Bacteria*, *Clostridia*, *Bacteroides* in faeces of vegetarians using qPCR and PCR-DGGE fingerprinting; Ann Nutr Metab. 2009 Jul 27;54(4):253-257.

Zwielehner, J., Liszt K., Handschur M., Lapin A., Haslberger A.; Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of Bacteroides, bifidobacteria and Clostridium cluster IV in institutionalized elderly. Exp Gerontol. 2009 Jun-Jul;44(6-7):440-6. Epub 2009 Apr 17.

They can be found in the appendix of this thesis. In these studies I also worked with other bacterial subgroups, but my main interest focused on *Clostridium* cluster IV.

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3. ABBREVIATIONS

A	Adenine
G	Guanine
T	Thymine
C	Cytosine
bp	base pairs
DGGE	Denaturing Gradient Gel Electrophoresis;
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mg	Milligram
PCR	Polymerase Chain Reaction
CFU	Colony forming units
min	Minute
mM	millimoles
μM	micromoles
pM	picomoles
vol	Volume
V	Volt
W	Watt
°C	degree Celsius
rRNA	ribosomal RNA

LB-agar	liquid broth medium
PBS	Phosphate buffered saline
FAM	6-carboxyfluorescein
BHQ	Black Hole Quencher
wt	weight
S_ab	sequence match score These are the number of (unique) 7-base oligomers shared between your sequence and a given RDP sequence divided by the lowest number of unique oligos in either of the two sequences.
FISH	Fluorescence in situ hybridization
CD	Crohn's diseases
UC	Ulcerative colitis
SCFA	Short chain fatty acids

4. Objectives

The human body lives in a symbiotic relationship with his gut microbes, which have an estimated mass of 1-2 kg and number of 10^{13}-10^{14} [LEY et al., 2006]. Furthermore, they seem to play an important role in health maintenance and diseases.

Members of *Clostridium* cluster IV are involved in beneficial functions of the human colonic microbiota such as carbohydrate breakdown, butyrate production [DUNCAN et al., 2002] and a proposed role in chronic inflammation status of the intestine [SOKOL et al., 2008], cancer [SCANLAN et al., 2008] and obesity [TURNBAUGH et al., 2009]. Clostridium cluster IV is one of the predominant bacterial subgroups in the human gut representing approximately a quarter of the faecal microbiota [LOUIS et al., 2007; SARTOR, 2008].

The amount of vegetarians rose in the last years and some studies associated a vegetarian diet with a decreased risk for heart diseases and various cancer types [VINNARI et al., 2008]. Some studies could show that life style factors in combination with a specific diet could influence the faecal microbiota [MUELLER et al., 2006; DICKSVED et al., 2007]. Until now to our knowledge only one investigation of vegetarian gut microbiota has been performed with molecular methods by investigating one vegetarian woman. Hayashi et al. reported that *Clostridium* cluster XIVa, *Clostridium* cluster IV, *Clostridium* cluster XVIII were the major components of the vegetarian gut microbiota [HAYASHI et al.].

During the ageing process a regression of physical functions occurs and physiologic anorexia, which is common in elderly, could lead to the development of cachexia. This includes a lesser food intake and malabsoprtion of dietary components. [MORLEY, 2007]

In addition, ageing is accompanied by impaired immune functions, which promotes infectious diseases. They are the underlying cause of mortality

beyond 75-80 years. Changes in the human microbiota in terms of composition, expression of virulent factors or metabolic activity affect the gut immune system. [GUIGOZ et al., 2008]

Mueller et al. reported significant decreased proportions of *Faecalibacterium prausnitzii*, a predominant member of *Clostridium* cluster IV, in Swedish and Italian elderly, while in the French group no marked differences could be showed and in German elderly slightly higher levels of this bacterium [MUELLER et al., 2006].

This diploma thesis aimed to investigate the qualitative and quantitative changes of the human intestinal microbiota focusing on *Clostridium* cluster IV already known as *Clostridium leptum* subgroup associated with vegetarianism and ageing. For this purpose, we used an approach with quantitative real time polymerase chain reaction (qPCR), PCR-denaturating gradient gel electrophoresis (DGGE), clone library and sequencing analysis.

5. Introduction and Literature Review

5.1. The human gut and his guests

The human body especially the human intestinal tract harbors a large, active and very complex community of microbes, which contain 100-fold more genes than the human genome does [HOOPER and GORDON, 2001; GILL et al., 2006]. Traditional methods like culturing methods cannot address all bacteria in the human gut, because the great part of them is strict anaerob and referred to be ‘uncultureable’. However, now molecular-based technologies permit genetic analysis of complex microbial populations without the need of cultivation.
Those new results estimate that there are approximately 800 different bacterial species with over 7000 strains, with an estimated mass of 1-2 kg and number of

10^{13}-10^{14}. Those numbers are based on the sequencing of the highly conserved 16S region of rRNA by Ley et al 2006.[LEY et al., 2006]

The number of these bacteria and fungi is increasing both in concentration and complexity from the proximal gastric, which is higher in aerobic organisms to the caecum and colon where predominantly anaerobic microorganisms live. Figure 1 shows the composition and luminal concentrations of dominant microbial species in various regions of the gastrointestinal tract. [SARTOR, 2008]

The majority of those microorganisms is non-pathogenic and they are called the commensal bacteria. Therefore, Ley et al thesis of a “superorganism” which should represent the humans close symbiotic association with the gut microbiota is largely accepted [LEY et al., 2006].

The gut microorganisms benefit from the constant nutrient flow, stable temperature and niches for various metabolic requirements provided by the intestinal environment.

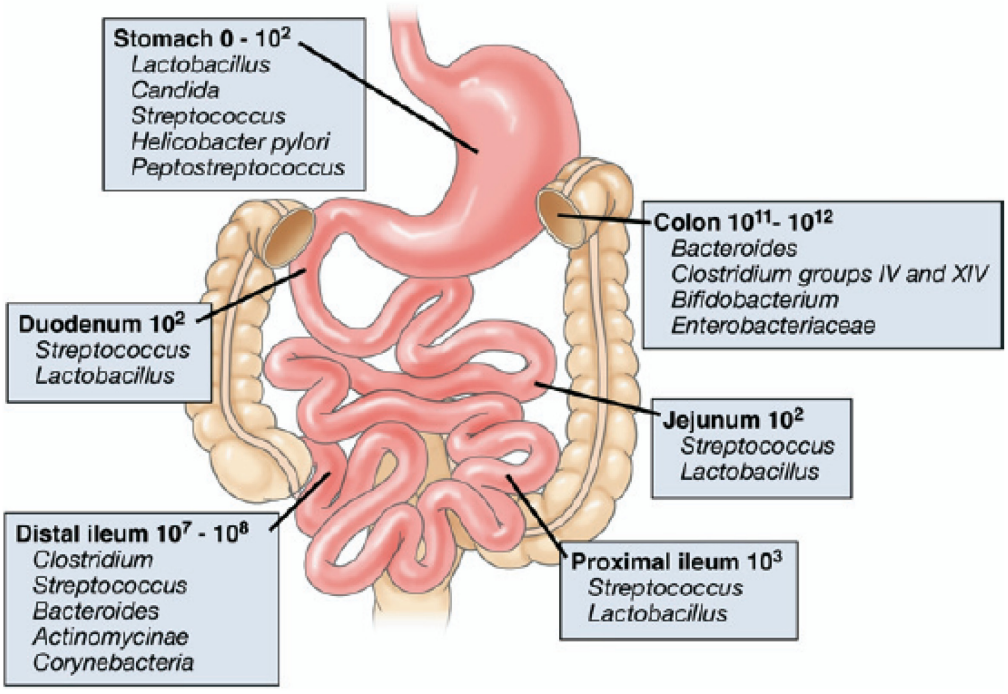

Figure 1 Composition and luminal concentrations of dominant microbial species in various regions of the gastrointestinal tract [SARTOR, 2008]

Gut microbes have the ability to digest residual dietary components in the large bowel. Those are complex polysaccharides as resistant starch, cellulose, hemicelluloses, pectins, gums, some oligosaccharides, unabsorbed sugars, sugar alcohols and proteins along with other secretions of the host [CUMMINGS et al., 1996]. Furthermore, they are able to produce Vitamin K and metabolize those materials to short chain fatty acids (SCFAs) like butyrate, propionate, acetate in lesser amounts formate, valerate and carproate [LIM et al., 2005]. SCFA are the favored energy source for colonocytes and those which are produced by bacteria account for up to 10% of the human energy source [SARTOR, 2008].

In addition, the intestinal microbiota provides an abundant source of potentially detrimental organisms, ligands, and antigens that can activate pathogenic innate and adaptive immune responses and metabolic products that affect epithelial and immune functions. [EWASCHUK and DIELEMAN, 2006] Figure 2 shows mechanisms of probiotics or commensal bacteria in the defense of epithelial cells.

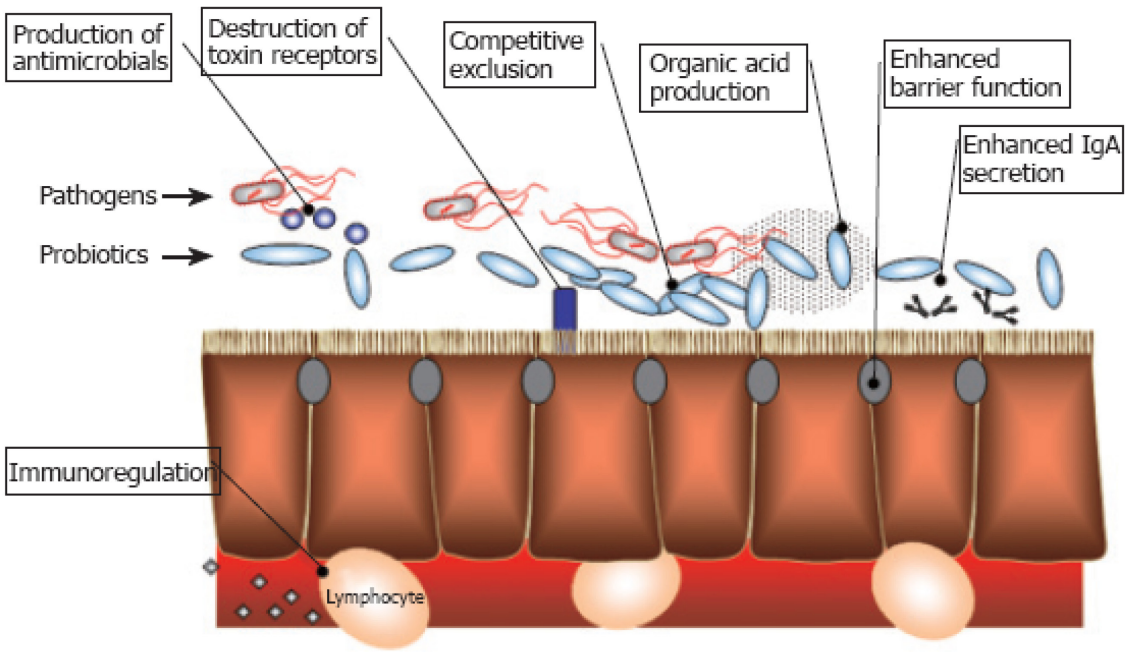

Figure 2 Mechanisms of probiotic activity [EWASCHUK and DIELEMAN, 2006]

5.2. Influence on the gut microbiota

Before birth the fetus gastrointestinal tract is a sterile environment. The first exposure of the infants gut to microbes occurs at the birthing process from the maternal faecal and vaginal flora and after a few month a relatively stable microbial population is established [FANARO et al., 2003]. Thus, we can say the first factor, which determine the composition of the microbiota is the mother's flora and the environment. However, also host genetic factors may influence interindividual variation in the intestinal microbiota as example via the availability of attachment sites and host-derived resources. Twin studies reported about a correlation between overall genetic relatedness and faecal community similarity [STEWART et al., 2005]. Host genotype certainly influences intestinal microbiota, but Stewart et al showed also that genetically identical individuals in very similar environments can have differences in their intestinal microbiota. [DETHLEFSEN et al., 2006]

One way to influence the intestinal microbiota is through diet, which affect the metabolism of microbes and consequently the human, as shown in Figure 3. Alterations in diet give different substrates resulting in fermentation via different metabolic routes and in the end in differed products, which can be metabolized by other bacteria and may support the growth of a bacterial group, which is also influenced by local conditions as pH, oxygen and hydrogen, metabolite concentration and gut transit time. [LOUIS et al., 2007]

Probiotics, prebiotics and synbiotics are used in functional foods or in therapeutic intervention to improve our health by influencing the gut microbiota. Bifidobacteria and lactic bacteria are commonly used as probiotics. Probiotics are able to survive stomach acid and bile and are safe for human consumption. Prebiotics are non-digestible dietary carbohydrates, which stimulate the growth and metabolism of endogenous gut bacteria. However, their proposed therapeutic effect in different diseases like atopic disease and inflammatory bowel diseases could not always be confirmed, the studies are contradictory

and further investigations are necessary. [EWASCHUK and DIELEMAN, 2006; VAEL and DESAGER, 2009]

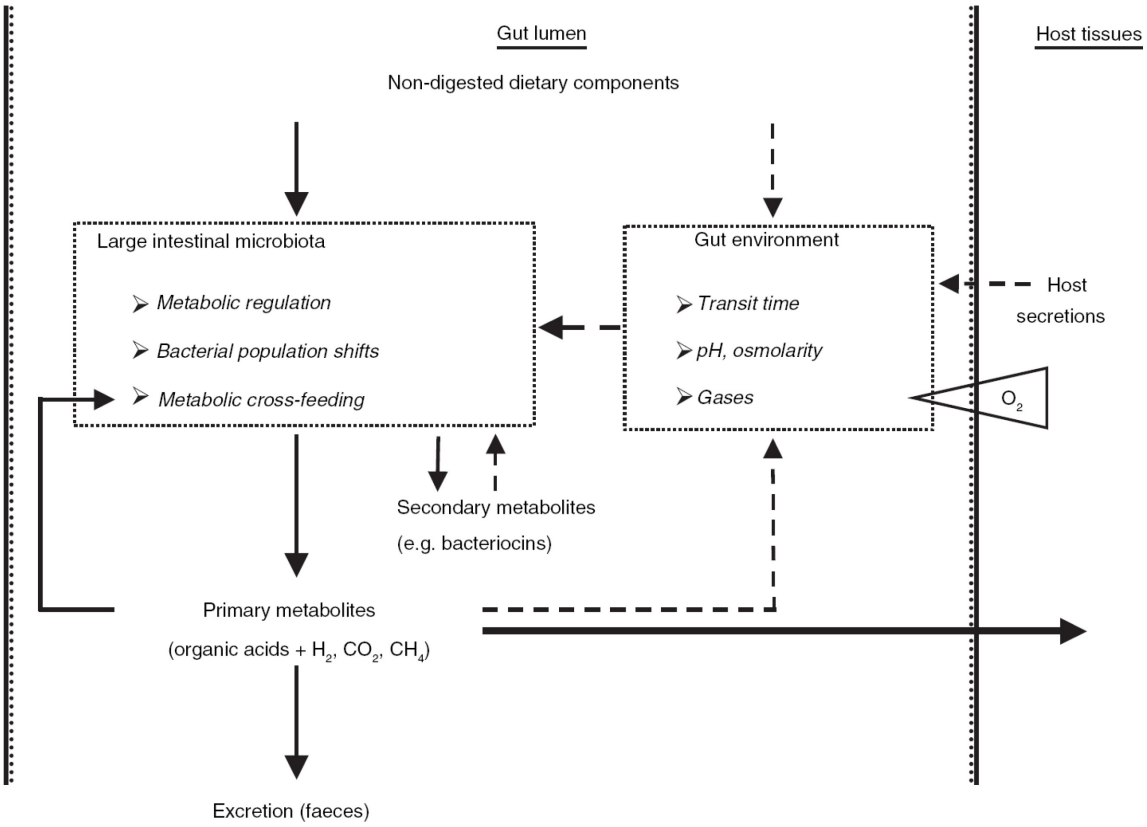

Figure 3 Schematic diagram of the gut microbial ecosystem.

Metabolic flows are shown with solid arrows as well as other influences are shown with dashed arrows. [LOUIS et al., 2007]

5.3. Human gut microbiota associated with diseases

The three major functions of the intestinal microbiota are metabolic, trophic and protective [VAEL and DESAGER, 2009]. Wikoff et al observed in their broad MS-based metabolomics study a large effect of the gut “microbiome” on mammalian blood metabolites [WIKOFF et al., 2009]. Therefore, the interaction of the human gut microbiota with diseases seems to be apparent.

5.3.1. Atopic disease in infants

It has been proposed that the initial composition of the infant microbiota is a key determinant in the development of atopic disease. A review by Penders et al. about the often contradictory literature on this subject observed that 14 out of 17 observational studies indicate an association between infant gut microbiota and atopic disease with a critical time window during the first 6 months of life. [PENDERS et al., 2007; VAEL and DESAGER, 2009]

5.3.2. Inflammatory bowel diseases

Crohn's disease (CD) and Ulcerative colitis (UC) are the two primary human inflammatory bowel diseases (IBD) and an association with an alteration of the human intestinal microbiota has been discussed and shown in different studies: Manichanh et al. did a metagenomic approach (clone libraries, sequencing, Fluorescence in situ hybridization (FISH)) with 6 healthy people and 6 patients with CD and found a significant reduction in Firmicutes in CD patients [MANICHANH et al., 2006]. Another study by Frank et al. detected a depletion in commensal bacteria, notably members of the phyla Firmicutes and Bacteroides in CD and UC patients by investigating 190 resected tissue samples from the small intestine from CD, UC and non-IBD control subjects in equal numbers [FRANK et al., 2007]. Sokol et al. investigated the composition of the mucosa-associated microbiota of 21 CD patients at the time of surgical resection and 6 months later using FISH analysis and observed that a reduction of Firmicutes, especially *Faecalibacterium prausnitzii* is associated with a higher risk of postoperative recurrence of ileal Crohn disease [SOKOL et al., 2008]. Another study by Sokol et al from 2006 reported decreased numbers of *Clostridium leptum* subgroup in CD patients compared to a healthy control group [SOKOL et al., 2006]. These studies indicate that Clostridium cluster IV is involved in IBD.

5.3.3. Cancer

The gut microbiota may also play a role in colorectal cancer. Metabolic activity of gut microbes which has been associated with an increased risk in colon cancer is the conversion of relatively benign compounds, such as bile acids, nitrate and plant glycosides into reactive metabolites, which are implicated in colorectal carcinogenesis. [LIM et al., 2005] An increased diversity of Clostridium leptum and C. coccoides subgroup were observed in colorectal cancer patients and polypectomized volunteers [SCANLAN et al., 2008].

5.3.4. Obesity

Symbiotic gut microbes are discussed to modulate human metabolic phenotypes which influence the weight-gaining or weight-loss process: Ley et al. investigated the relation between gut microbial ecology and body fat in human by studying 12 obese people, over the course of 1 year by sequencing 16S ribosomal RNA genes from stool samples. They could show a dynamic linkage between adiposity and microbial ecology and a higher amount of *Firmicutes* and lesser numbers of *Bacteroidetes* in obese people than in lean. [LEY et al., 2006] Turnbaugh et al. investigated 154 individuals (54 twin pairs and of some of them their mothers) with a new sequencing approach and found out that obesity is associated with phylum-level changes in the microbiota, reduced bacterial diversity and altered representation of bacterial genes and metabolic pathways. [TURNBAUGH et al., 2009]

5.4. Microbiota

More than 99% of the gut microbiota can be divided into four bacterial divisions, the Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. The greatest division of them is those of the Firmicutes (64% of colonic species), which is mainly composed of the Clostridium cluster XIV and Clostridium cluster IV group [SARTOR, 2008].

5.4.1. Clostridium cluster IV/Clostridium leptum subgroup

Therefore some studies could show that in average a quarter of the human gut microbiota belongs to the Clostridium cluster IV group [LOUIS et al., 2007].

The members of *Clostridium* cluster IV are phenotypically heterogenous, it includes both mesophiles and thermophiles. Furthermore, it exhibits a broad chromosomal DNA G+C content range between approximately 27 to 52 mol%. The gram-positive *Clostridium leptum* subgroup includes certain members of the genera *Clostridium*, *Ruminococcus*, *Eubacterium* and *Faecalibacterium.* [COLLINS et al., 1994] However, the most frequent member of Cl. cluster IV is *Faecalibacterium prausnitzii* followed by the *R. bromii* species and relatives [SUAU et al., 2001; LAY et al., 2005].

Members of *Cl. leptum* subgroup are highly oxygen sensitive anaerobes and therefore difficult to culture. Thus, the development of non-culturing molecular methods could give us new insights into the *Cl. leptum* group. Furthermore, this cluster contains butyrate-producing and fibrolytic bacteria [DUNCAN et al., 2002] and thereby contribute to processes important to colonic health, because butyrate is the preferred energy source for the colonocytes. The most significant n-butyrate-producing gut bacterium is *Faecalibacterium prausnitzii.* The variation of *Faecalibacterium prausnitzii* population is associated with modulation of 8 urinary metabolites of diverse structure. This indicates that this species is a highly functionally active member of the microbiome, influencing numerous pathways. [LI et al., 2008]

Furthermore, members of *Clostridium* cluster IV seem to play an important role in diseases like inflammatory bowel diseases [SOKOL et al., 2008], colorectal cancer [SCANLAN et al., 2008], obesity [LEY et al., 2006] and therefore, further investigations about this group are needed.

5.4.2. Bacteroides

Another important group (23 % of species in the gut) is the Bacteroides group [SARTOR, 2008]. Bacteroides are anaerobic, gram-negative, non-sporulating bacteria. They are involved in carbohydrate fermentation and some of them are also butyrate-producing and fibrolytic bacteria. The ubiquitious member *Bacteroides thetaiotaomicron* is known to be involved in beneficial functions, including nutrient absorption, epithelial cell maturation and maintenance. [HOOPER and GORDON, 2001; ECKBURG et al., 2005]

5.4.3. Bifidobacteria

Bifidobacteria are gram-positive bacteria and belong to the division of Actinobacteria. They are also a dominant group of the human gut and their relative abundance in faecal samples has been reported to be between 2.5 to 4.9 [LOUIS et al., 2007]. In the first year of life in brest-fed children the human gut is mainly dominated by bifidobacteria and therefore seems to be important in the development of the immune system [VAEL and DESAGER, 2009]. Bifidobacteria are commonly used as probiotics in food industry. They are also able to ferment non-digestible dietary carbohydrate to short chain fatty acids. Furthermore, it has been reported that bifidobacteria are involved in the prevention of atopic disease [KIRJAVAINEN et al., 2001] and in the prevention of obesity and insulin resistance via an enhanced barrier function of the gut epithelium [CANI et al., 2007].

5.5. Vegetarianism

Vegetarianism (e.g., little or no animal protein, low fat and high fibre content [VINNARI et al., 2008]) increased substantially during the last few decades in the Western world and this trend is likely to continue in the future [2003; VINNARI et al., 2008]. Vegetarianism has been associated with decreased risk for diseases such as heart diseases, various cancers and has been linked to a lower BMI and an overall decline in mortality [VINNARI et al., 2008]. Nevertheless, vegetarians could also have a malabsorption syndrome, through a high phytate intake. Famularo et al. postulated that manipulating the endogenous digestive microflora of vegetarians through administering probiotic lactic bacteria would represent an innovative tool to counteract the occurrence of the malabsorption syndrome. [FAMULARO et al., 2005]
Different lifestyle factors and diets were shown to have a significant impact on the faecal microbiota [MUELLER et al., 2006; DICKSVED et al., 2007; LEY et al., 2008]. However, until now the microbiota of vegetarians has marginally investigated with molecular methods and only few studies have been published.

Alm et al. investigated the influence of an antroposphic lifestyle (diet comprising fermented vegetables, restrictive use of antibiotics, anti-pyretics and vaccinations) on the intestinal microflora in infancy. Therefore they compared children with an antroposophic lifestyle with children with a traditional lifestyle. 59.4 % in the antroposophic group were vegetarians. They could show that the number of *Enterococci* was significantly higher in breastfed and vegetarian infants. Furthermore, infants with an antroposophic lifestyle had a higher proportion of acetic acid and a lower proportion of propionic acid in their stool as compared to control children. In conclusion, they could show that lifestyle factors influence the composition of the gut flora in infants. Nevertheless, they compared a group of lifestyle factors and did not concentrate on vegetarianism. [ALM et al., 2002]

To our knowledge only one investigation [HAYASHI et al.] of vegetarian microbiota has been performed with molecular methods. Hayashi et al.

analysed the faecal microbial diversity in a strict vegetarian woman and revealed that *Clostridium* cluster XIVa, *Clostridium* cluster IV, *Clostridium* cluster XVIII were the major components of the vegetarian gut microbiota [HAYASHI et al., 2002].

5.6. Ageing

Nowadays we live in a society where people becoming older as ever before and this trend is likely to continue. In western societies people over 60 currently constitute one-fifth of the population. Advancements in science and medicine, as well as improved living standards made an increased life expectancy possible. Therefore the need for more healthcare resources is evident. However, what does ageing mean? It can be defined as '*the regression of physiological function accompanied by advancement of age'*. [WOODMANSEY et al., 2004]

Furthermore, the question is how ageing affects the gastrointestinal tract, because this knowledge is important in the treatment and prophylasxis of diseases, and in maintenance of health.

The obvious change is the physiologic anorexia of ageing, which could lead to the development of cachexia. However, this involves multiple little changes, like taste and smell, altered fundamental compliance, altered secretion of gastrointestinal hormones, alterations in autonomic nervous system feedback to the central nervous system, alterations in the fat hormone leptin and in steroid hormones, and changes in the central nervous system in response to food intake. [MORLEY, 2007]

With ageing there is a marked decrease in olfactory ability resulting in foods taste bland and uninteresting. This is coupled with masticatory dysfunction caused loss of teeth and muscle bulk and swallowing difficulties which can lead to an imbalanced diet. [WOODMANSEY, 2007]

Furthermore, older people are earlier satiated, because there is a decline in the adaptive compliance of the fundus of the stomach. Therefore, food escapes

more rapid from the fundus to the antrum. That means that the antral stretch occurs earlier, resulting in early satiation. Another factor which leads to the physiologic anorexia is the alteration of the secretion of gut hormones. It could be detected that there is an increased release of cholecystokinin (CCK), which acts as a satiety hormone, in aged people. Another very important satiation hormone, Leptin, also increases with ageing, because of the decline in testosterone. That's way maybe anorexia occurs more in ageing man than in woman. Gastric acid and pepsinogen secretion increase with aging and this is also associated with an increase in gastric mucosa proliferation. Further observations showed that postprandial hypotension, dysphagia and the associated aspiration pneumonia occur with increasing frequency as we age. Migratory motor complexes are not different between young and old people, but an increase in colon transit time occurs with ageing due to an increase in colon transit time. This results in faecal impaction and constipation, which is a major problem in elderly people. [MORLEY, 2007]

Woodmansey et al 2004 reported a reduction in faecal weight with increased age [WOODMANSEY et al., 2004]. Another study of Stephen et al 1987 correlated low faecal weights to slow intestinal transit times and reduced excretion of bacterial matter [STEPHEN et al., 1987]. Increased retention time is associated with an increase in bacterial protein fermentation and therefore also with the levels of ammonia and phenols generated in putrefactive processes in the gut. [WOODMANSEY, 2007]

In sum, those physiological changes in the gastrointestinal tract, as well as modifications in diet and host immune system activity affect the composition and metabolism of the gut microbiota and in turn affect the individual.

The most remarkable changes in the ageing gut are diminished numbers of bifidobacteria and an increase in Enterobacteria, while in other bacterial groups the studies are contradictory [WOODMANSEY, 2007; GUIGOZ et al., 2008]. Mueller et al reported in a population cross-sectional study with relevant numbers of healthy subjects, 85 adults (age 24-49 years) and 145 elderly (age 61-100 years) from four European countries (France, Germany, Italy and

Sweden) significant decreased proportions of *Faecalibacterium prausnitzii* in Swedish and Italian elderly, while in the French group no marked differences could be showed. The group of German elderly showed slightly but not significantly higher levels of this bacterium. Furthermore, they reported significantly higher proportions of Enterobacteria in elderly independent of the location, but bifidobacteria only tended to be lower in elderly. In Italian elderly they detected a decrease of the *Bacteroides-Prevotella* group, while in German elderly the number of the same group increased. In sum, Mueller et al could detect age-related differences in the microbiota but they also differ between the study populations from the four countries, where each showed a characteristic colonization pattern. [MUELLER et al., 2006]

Another interesting study by Bartosch et al compared healthy elderly volunteers living in the local community, elderly hospitalized patients and elderly hospitalized patients receiving antibiotic treatment, using DNA extracted from feces and real-time PCR. They reported a marked reduction of *Bacteroides-Prevotella* group following hospitalization and also reductions in bifidobacteria, *Clostridium clostridiiforme* and *Faecalibacterium prausnitzii* in hospitalized patients. However, the relative abundance (percentage of the subgroup to the total bacterial load) of the last three groups did not change. Antibiotic treatment resulted in further reductions of certain bacterial communities and in some patients to a complete elimination. [BARTOSCH et al., 2004]

6. Material and Methods

6.1. Study design

Figure 4 gives a short overview of the study design and the used methods in this diploma thesis and therefore in the two published articles. As you can see in the both articles also bifidobacteria and Bacteroides have been investigated, but my main work consisted in establishing a system for analyzing *Clostrdium*

cluster IV. Therefore, the methods, results and discussion concentrate on this cluster.

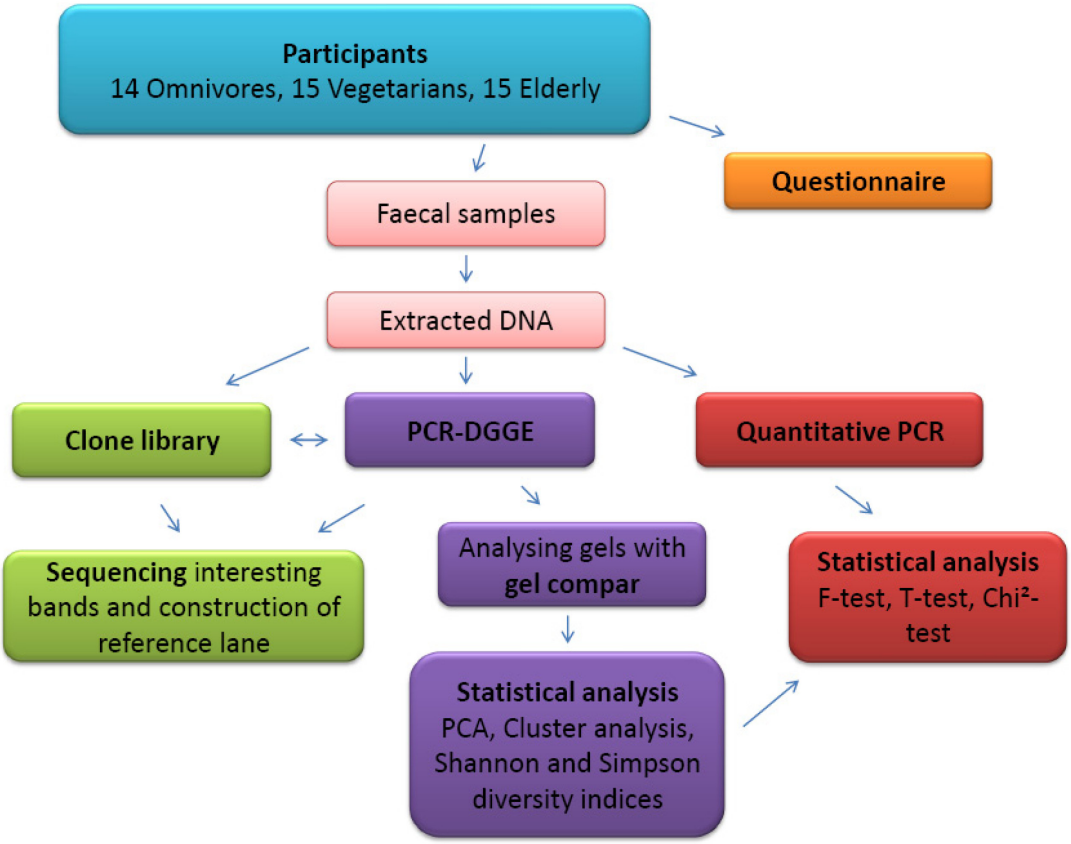

Figure 4 Study design of this thesis.

6.2. Participants

Fifteen institutionalized elderly aged 86±8 years, BMI 21.75 ± 5.08, from a geriatric department in Vienna and 29 students from Vienna joined the study. Nine patients were bed-ridden and six mobile. Geriatric patients did not consume any whole grain products at all but received supplements with soluble fibre (Benfiber®, Novartis). Fifteen young volunteers aged 23.2 ± 2.4 years, BMI 20.44 ± 1.77 followed a vegetarian diet and 14 individuals, aged 24±2.5 years,

BMI 22.68 ± 3.41, adhered to a typical Central European diet, called omnivores. 20 % of the vegetarians assessed followed vegan diet. Only participants who did not receive antibiotic or chemotherapeutic treatment three months prior to sampling were included in the study. A questionnaire about dietary habits and health activities was given to all participants.

6.3. Sampling and DNA Exraction

Stool samples were immediately stored at –20°C after sampling. 200 mg frozen stool sample were homogenized twice for 45 sec in a bead beater (Mini-Beadbeater-8) with lysis buffer. DNA was extracted using the DNA Stool Mini Kit (Qiagen) following the manufacturers' protocol with minor modifications and immediately stored at –20°C. The DNA extraction of all samples has been done by my colleague Selen Irez with my support.

6.4. Clone library for standard and reference lane

A clone library was constructed from 16S rDNA fragments obtained with primer pair 27f and sg-Clept-R3 specific for members of the *Clostridium* cluster IV. PCR products were inserted into a p-GEM Easy Vector (Promega) following the instructions of the manufacturer. The clone library was screened as described elsewhere [SONG et al., 2004]. Reamplified and purified amplicons were sequenced by Confidence DNA-Analysen GmbH (Vienna). Nucleotide sequences were corrected for vector and primer sequences in CodonCode Aligner (www.codoncode.com) and compared to previously published sequences in RDP 9.58 (http://rdp.cme.msu.edu/). A reference marker containing 16S rDNA fragments of seven clones from faecal material was loaded to each DGGE-gel in duplicate to allow gel-to-gel comparison in the PCR-DGGE analysis.

Clone CL16 was aerobically cultivated on LB-agar (liquid broth medium). The biomass was resuspended in sterile phosphate buffered saline (1x PBS from 10x PBS Roti®stock, ROTH). Tenfold dilutions from this suspension in sterile 1x

PBS were plated in duplicate on LB agar, colony forming units (CFU/ml) were counted and DNA extracted from serial dilutions. DNA was extracted from clone 16 using the Wizard® Plus.SV Minpreps DNA Purification System (Promega).

Table 1 Primer used for the establishing of the clone library

Target	Primer	Sequence	Reference
Bacteria	27f	GTGCTGCAGAGAGTTTGATCCTGG CTCAG	[EDWARDS et al., 1989]
Flanking regions of cloning vector	T7	TAATACGACTCACTATAGGG	Promega
	SP6	ATTTAGGTGACACTATAGAATAC	Promega

6.5. TaqMan qPCR

The TaqMan-assay was carried out in a Rotorgene 3000 (Corbett Life Science). Each reaction was done in duplicate in a volume of 10 µl containing 5 µl TaqMan SensiMix DNA Kit (Quantace), 1 µl of each primer and probe (table 1) and 2 µl of the 100-fold dilution of the template. All probes were labeled with 6-FAM at the 5' end and carried a BHQ-1 quencher at the 3' end. TaqMan Probe (Clep-P) for *Clostridium* cluster IV was designed with CLC DNA Workbench. Analysis with ProbeMatch (rdp.9.58) resulted in the finding that Clep-P binds to all members of *Clostridium* cluster IV. The amplification program for *Clostridium* cluster IV and the universal primer were: denaturation at 95° for 5 min and 45 cycles at 95°/ 55°C for 30/ 45 sec.

DNA of clone CL16 and one faecal sample were used to construct standard curves for comparison of PCR reaction efficiencies among different experiments and enumeration of all bacterial groups. Reaction efficiency (E) was estimated using the slope of the standard curve and the formula $E=10^{(-1/slope)}$ -1 as described elsewhere [YOUNGSEOB, 2005]. Quantisation was done using standard curves obtained from known concentrations of organisms containing the respective amplicon for each set of primers. The percentage of bacterial

group rRNA gene copies in relation to total rRNA gene copies (relative abundance) was calculated for each individual, and the mean was determined for each subject group. Relative quantification (% of bacteria) was performed using Rotor-Gene 3000 calculation software (Corbett operator manual) and Excel.

Formulas used in Rotor-Gene 3000 for the evaluation of concentration:

$C=n \times E^{ct}$

$E= -1+10^{(-1/slope)}$

C …Concentration

n… initial copy numbers

E… Efficiency

Ct…cycle number

Table 2 Primers and probes used for quantification of faecal bacteria using TaqMan assays targeting 16S rRNA coding regions.

Target	Primer pair and Probe	sequence (5' - 3')	Size (bp)	Conc. [µM]	Reference
All bacteria	BAC-338-F	ACTCCTACGGGAGGCAG	468	10	[YU et al., 2005]
	BAC-805-R	GACTACCAGGGTATCTAATCC		10	
	BAC-516-P	(FAM)-TGCCAGCAGCCGCGGTAATAC-(BHQ-1)		2	
Clostridium cluster IV	sg-Clept-F	GCA CAAGCAGTGGAGT	239	4	[MATSUKI et al., 2004]
	sg-Clept-R3	CTTCCTCCGTTTTGTCAA		4	
	Clep-P^{++}	(FAM)-AGGGTTGCGCTCGTT-(BHQ-1)		2	This study

$^{++}$ position of target site (numbering corresponding to *E . coli* 16S rRNA gene) 1082 to 1107.

6.6. PCR, DGGE fingerprinting

16S rRNA coding regions were amplified using a ready-to-use mastermix (Promega). Bovine serum albumin (10mg/ ml, Fermentas) was added to a final concentration of 0.2 μg/ µl. Primer concentration in the reaction volume was 0.5 µM and $MgCl_2$-concentration was 2 mM. The amplification was carried out in a Robocycler (Stratagene). Fragment length was checked on a 2% ($wt*vol^{-1}$) agarose gel and visualized under UV light after ethidium-bromide staining (0.5 $mg*ml^{-1}$). For DGGE analysis, PCR was performed in 2 x 50 μl reaction volume with primer pair sg-Clept-R3 with a GC-clamp at the 5' end and sg-Clept-F [MUYZER et al., 1993] as described elsewhere [LEY et al., 2006] and precipitated over night at –20°C.

The reference lane for DGGE fingerprinting consisted of CL2, CL10, CL12, CL16, CL18, CL22 and CL26. Those clones have been sequenced as you can see in the results of sequencing analysis (Table 5).

DGGE gels were prepared as described previously [FORSYTHE et al., 2009] with a linear gradient of 30-50% using a gradient mixer (Hoefer SG 30) and a peristaltic pump.

6.7. Statistical analysis

Student's t-tests (Excel) were performed to compare the q-PCR data of each group. The equality of variances was tested with the F-test as implemented in Excel. Student's t-tests and F-test were also performed on the Shannon and Simpson results generated from the DGGE-fingerprints to establish if any significant differences were evident between the diversity of the three groups. The used DGGE-fingerprint data were given by presence or absence of bands in order to rule out PCR bias. Food frequency data were analyzed based on Chi-square approximation as implemented in SPSS. P values <0.05 were considered significant. DGGE fingerprints were compared using GelComparII

(www.applied-maths.com). Band comparison tables were analyzed with principal component analysis (PCA) using the default settings in 'R-software environment for statistical computing' (www.r-project.org) until 100% variance was explained. Transformed data were plot in a bi-plot as a function of the first two principal components. Clustering was applied to DGGE fingerprinting data as implemented in the GelComparII environment. Clustering was performed based on Dice coefficient, which takes band positions into account. Jackknife analysis was performed using average similarities and 100 resamplings. Jackknife analysis is a leave-one-out method that tests the reliability of the clustering similar to bootstrap analysis. Shannon and Simpson's diversity index were calculated on binary band information (presence-absence) with the default settings implemented in the 'vegan' package in 'R'. Shannon index is defined as $H = -\sum p_i \ln p_i$, where p_i is the proportional abundance of species i. For Simpson's index D, this is 1-sum((p_i)^2), where p_i is the relative frequency of the i-th species.

7. Results

7.1. Dietary aspects

Analysis of the participants' dietary habits indicated similar consumption patterns of liquids, alcohol, fruits, grains and milk products in all groups. Exercise levels were comparable. Omnivores stated significantly less frequent (Chi^2 Test; $p<0.027$) consumption of vegetables than vegetarians. Three of the vegetarians assessed followed a vegan diet; all others followed a lacto-ovo vegetarian diet. Five vegetarians stated to eat fish a few times a year. Omnivores stated significantly less frequent (Chi^2 Test; $p<0.04$) consumption of meat than elderly participants and regular consumption of whole grain products several times a week. The institutionalized elderly of this study did not consume any whole grain products at all but received supplements with soluble fiber (Benfiber®, Novartis).

7.2. TaqMan-quantification

Members of *Clostridium* cluster IV were quantified as percentage of the total bacterial DNA (Figure 5). No cross reactivity of group-specific primers and probes with non-target strains could be detected. Test-retest variations were between 2.7 % and 5.2 %, values after relative quantification varied for less than 4 %. Vegetarians showed 19 % higher and elderly showed 26 % lesser counts of bacterial DNA than omnivores but these differences were not significant due to high interindividual deviations. The mean proportion of *Clostridium* cluster IV in stool samples of vegetarians was 31.86 ± 17.00 %, in omnivores 36.64 ± 14.22 % and in elderly 27.20 ± 14.63. The three vegans did not stand out in the group of vegetarians.

Table 3 Calculated concentrations and percent of all samples (1:100 diluted) in copies/reaction evaluated by real time PCR.

Samples 9v, 36v and 32v followed a vegan diet.

Vegetarians	Cl. clust. IV	Bacteria	Ratio	**in %**
1v	342,00	810,00	0,42	42,00
2v	80,00	315,00	0,25	25,00
22v	54,00	79,00	0,69	69,00
5v	788,00	2686,00	0,29	29,00
6v	767,80	1672,00	0,46	46,00
7v	926,25	4787,20	0,19	19,00
9v	447,06	1953,72	0,23	23,00
20v	188,29	1211,40	0,16	16,00
21v	37,10	234,79	0,16	16,00
23v	3,01	939,43	0,00	0,00
36v	1160,48	3045,33	0,38	38,00
38v	1744,02	3546,36	0,49	49,00
45v	462,80	2649,12	0,17	17,00
24v	265,13	799,22	0,33	33,00
32v	445,37	1852,55	0,24	24,00
mean:	**514,09**	**1893,01**	**0,30**	**29,73**
st. dev.:	**488,31**	**1320,71**	**0,17**	**17,00**
st. dev. %:	**94,99**	**69,77**	**57,18**	**57,18**

Omnivores	Cl. clust. IV	Bacteria	Ratio	**in %**
3m	127,00	300,00	0,42	42,00
4m	978,00	2492,00	0,39	39,00
11m	307,00	1483,00	0,21	21,00
40m	586,00	1060,00	0,55	55,00
43m	78,00	221,00	0,35	35,00
17m	363,77	928,70	0,39	39,00
18m	331,18	1057,63	0,31	31,00
19m	357,95	1100,25	0,33	33,00
33m	484,45	2324,73	0,21	21,00
34m	631,56	3822,33	0,17	17,00
35m	1233,88	2528,30	0,49	49,00
39m	1528,23	2959,79	0,52	52,00
41m	891,71	1452,20	0,61	61,00
42m	88,60	504,64	0,18	18,00
mean:	**570,52**	**1588,18**	**0,37**	**36,64**
st. dev.:	**440,63**	**1077,09**	**0,14**	**14,22**
st. dev.%:	**77,23**	**67,82**	**38,81**	**38,81**

Elderly	Cl. clust. IV	Bacteria	Ratio	**in %**
8g	41,00	79,00	0,52	52,00
16g	442,00	1292,00	0,34	34,00
10g	717,00	4325,00	0,17	17,00
12g	423,00	2029,00	0,21	21,00
13g	424,95	1954,62	0,22	22,00
14g	57,89	540,72	0,11	11,00
15g	86,81	1296,02	0,07	7,00
25g	231,18	969,46	0,24	24,00
26g	364,72	1589,24	0,23	23,00
27g	748,86	1840,41	0,41	41,00
28g	641,47	1275,22	0,50	50,00
29g	81,23	375,15	0,22	22,00
31g	204,91	539,29	0,38	38,00
37g	84,28	1519,93	0,06	6,00
44g	596,07	1489,92	0,40	40,00
mean:	**343,03**	**1407,67**	**0,27**	**27,20**
st. dev.:	**251,61**	**1000,29**	**0,15**	**14,63**
st. dev. %:	**73,35**	**71,06**	**53,79**	**53,79**

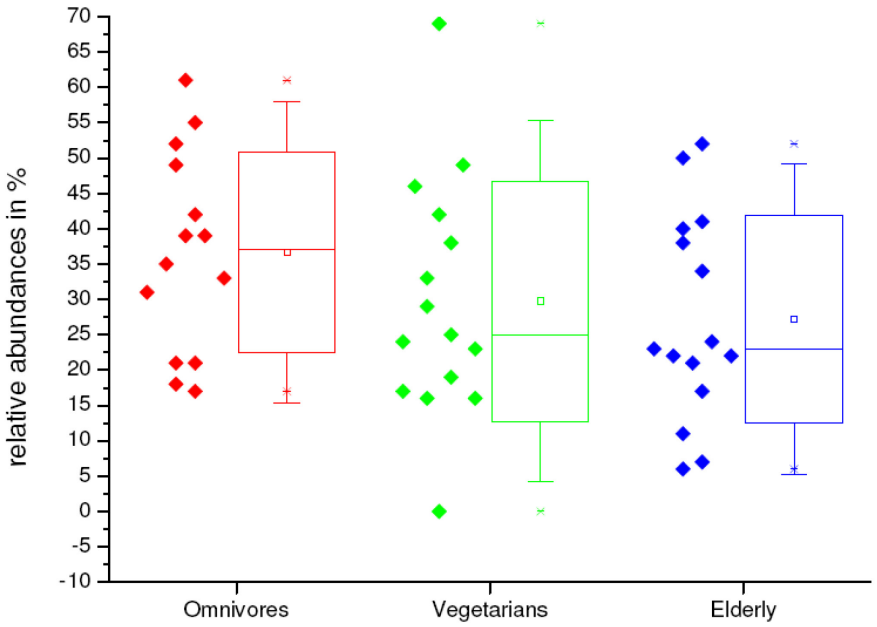

Figure 5 Proportions of *Clostridium* cluster IV of total bacterial load in stool samples of omnivores, vegetarians and elderly.

red, Omnivores; green, Vegetarians; blue, Elderly.

7.3. PCR-DGGE fingerprinting

Figures 6 to 10 show the PCR-DGGE fingerprints of all samples, which have been analysed with Gelcompar II.

The mean numbers of bands were 12.86 ± 3.01 for omnivores, 12.64 ± 3.82 for vegetarians and 10 ± 3.53 for elderly.

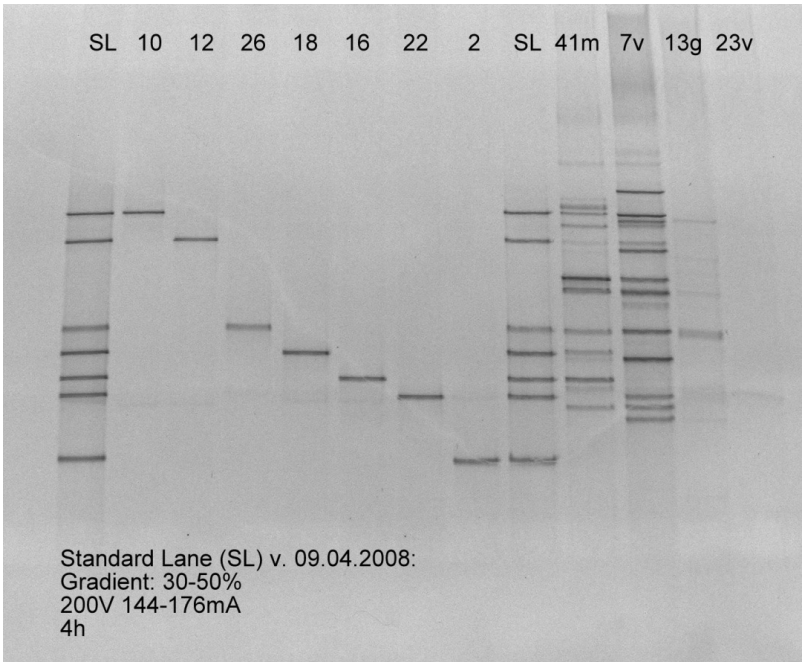

Figure 6 PCR-DGGE bandpattern of 16S rRNA coding regions of *Clostridium* cluster IV. Standard lane consisting of clones 10, 12, 26, 18, 16, 22, 2 used as reference marker for all PCR-DGGE fingerprints.

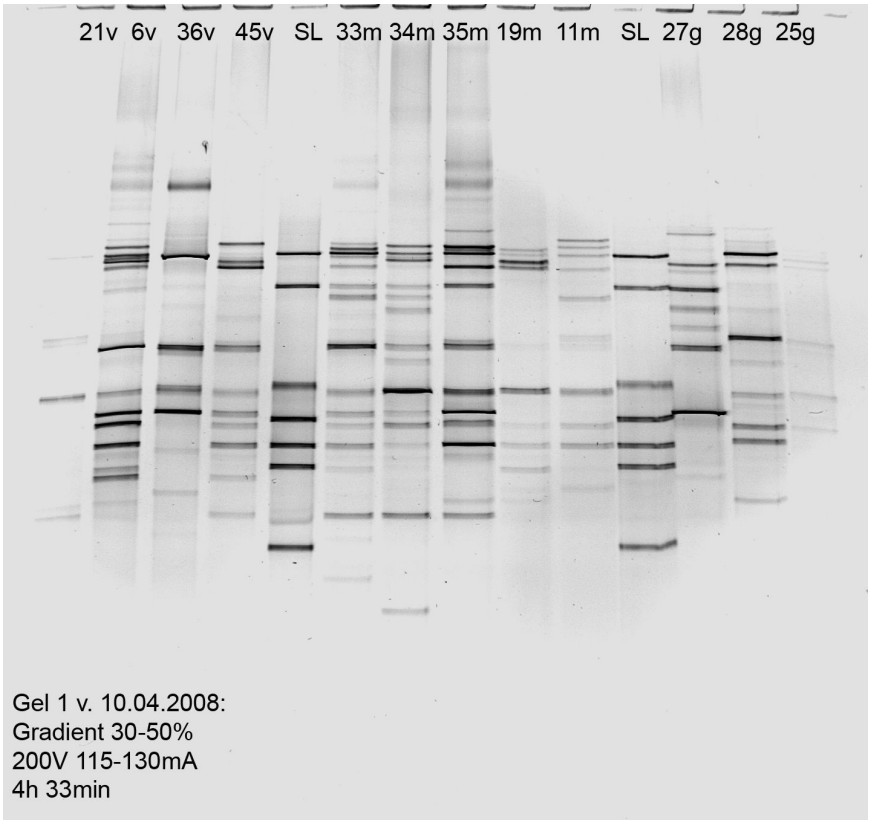

Figure 7 PCR-DGGE bandpattern of 16S rRNA coding regions of *Clostridium* cluster IV, Gel 1.

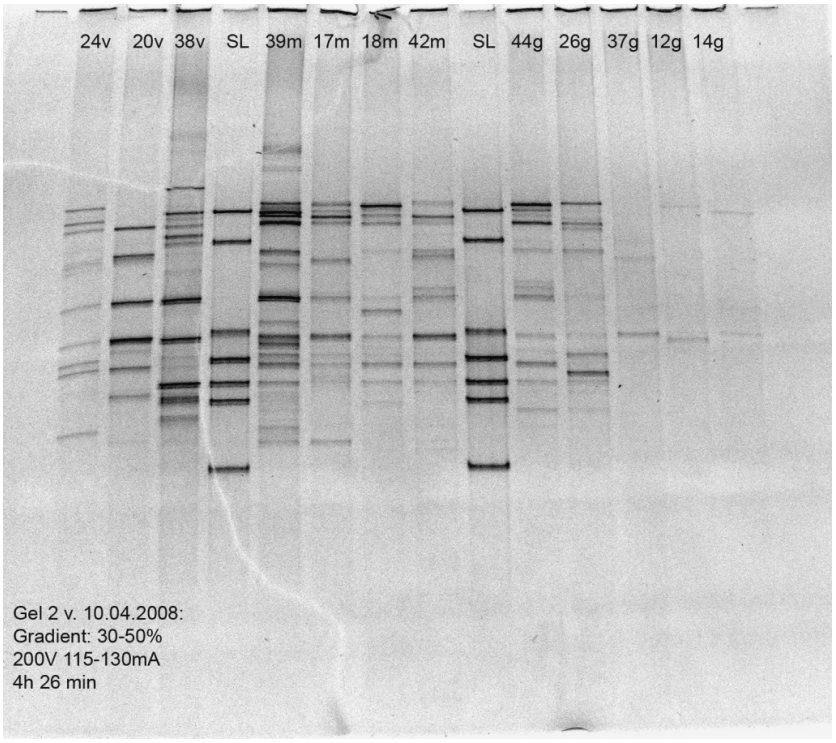

Figure 8 PCR-DGGE bandpattern of 16S rRNA coding regions of *Clostridium* cluster IV, Gel 2.

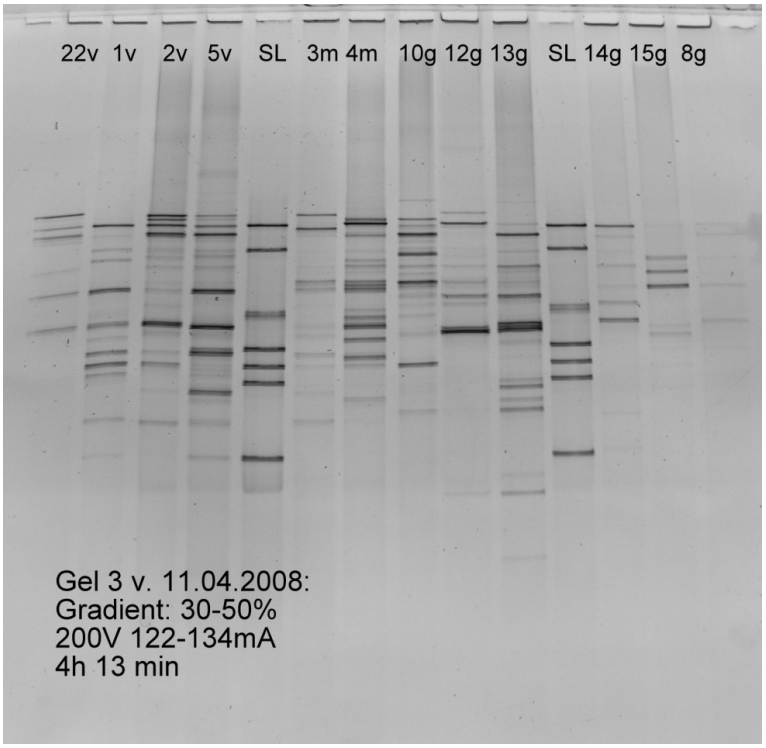

Figure 9 PCR-DGGE bandpattern of 16S rRNA coding regions of *Clostridium* cluster IV, Gel 3.

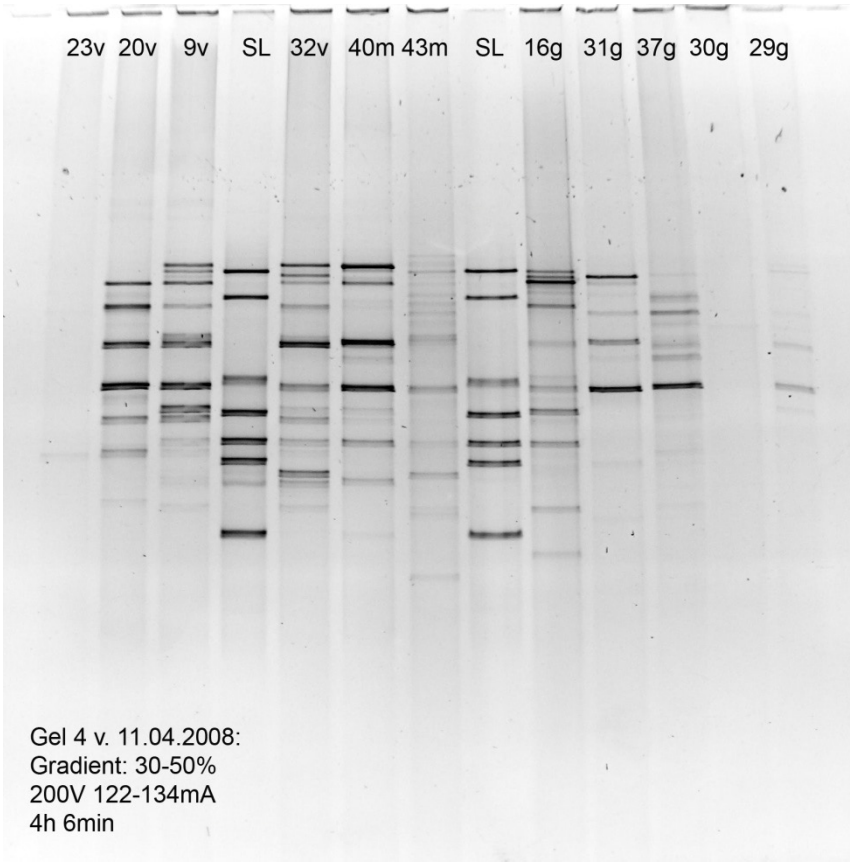

Figure 10 PCR-DGGE bandpattern of 16S rRNA coding regions of *Clostridium* cluster IV, Gel 4.

7.3.1. Sequencing analysis and interesting bands

Two bands (Table 4) from *Clostridium* cluster IV were more prevalent in omnivores than in vegetarians and elderly. The first sequence (band 833,31) was 96.7 % similar to *Faecalibacterium prausnitzii*^{T} and 99.5 % to *Faecalibacterium prausnitzii* AJ270469. The second sequence (band 723,75) matched next to *Clostridium* sp. BI-114^{T} (similarity 94.7 %) and uncultured bacterium, identified in a human faecal sample, DQ793301 (similarity 97.9 %) assigned to the familiy *Ruminococcaceae* [VAN DER WAAIJ et al., 2005]. One band (790.93) was significantly more prevalent in omnivores than in elderly. This sequence had the highest similarity (97.5 %) to Clostridium sp. BI-114^{T}. Another band (620.75) was significantly more prevalent in elderly than in vegetarians and for a tendency in omnivores. This band could be assigned to an uncultured bacterium DQ793399 with 98.0 % similarity.

Table 4 Appearance of different bands in the PCR-DGGE fingerprints in the 3 study groups.

Band	458.41	490.49	620.75	723.75	790.93	833.31
Vegetarians	7	7	2	5	7	6
Omnivores	8	12	4	11**	10*	13**
Elderly	10	7	9*	4	3	4

**, Significant ($p<0.05$) to both other groups; *, significant ($p<0.05$) to one of the other groups.

Table 5 Matching sequences from RDP 9.58 of clones and interesting bands.

Violet highlighted numbers indicate the similarity score, orange highlighted numbers are the S_ab score (seqmatch score).

Clone	Bandname	Type Strain	Similar sequence
Cl 10	460	0.966 0.853 1385 Faecalibacterium prausnitzii (T); ATCC 27768; AJ413954	0.992 0.956 1391 uncultured bacterium; SJTU_A2_05_52; EF403698
	458,41	0.959 0.774 1256 Ruminococcus bromii (T); ATCC 27255; L76600	0.990 0.995 1378 uncultured bacterium; from human colonic sample; HuCB2; AJ408987
Cl 12	525	0.978 0.874 1385 Faecalibacterium prausnitzii (T); ATCC 27768; AJ413954	0.989 0.925 1322 uncultured bacterium; RL188_aah15c06; DQ802261
Cl 26	710	0.929 0.703 1280 Eubacterium desmolans (T); L34618	0.975 0.825 1400 uncultured bacterium; 001B-f6; DQ904799
Cl 18	775	0.959 0.773 1326 Clostridium sp. BI-114 (T); type strain:BI 114=CCUG 47106=DSM 15176; AJ518869	0.992 0.886 1263 uncultured bacterium; C335; DQ326344
Cl 16	830	0.967 0.789 1385 Faecalibacterium prausnitzii (T); ATCC 27768; AJ413954	0.984 0.856 1308 uncultured bacterium; RL199_aaj42b01; DQ793312
	833,31	0.922 0.765 1385 Faecalibacterium prausnitzii (T); ATCC 27768; AJ413954	0.995 0.951 1389 Faecalibacterium prausnitzii; A2-165; AJ270469
Cl 22	870	0.921 0.718 1280 Eubacterium desmolans (T); L34618	0.972 0.951 1321 uncultured bacterium; RL201_aai45e05; DQ801686
Cl 2	1030	0.818 0.377 1385 Faecalibacterium prausnitzii (T); ATCC 27768; AJ413954	0.823 0.406 1270 uncultured bacterium; L729; AY981568
	490,49	0.899 0.595 1416 Ethanoligenens harbinense (T); YUAN-3; AY295777	0.995 0.995 1272 uncultured bacterium; D005; AY916350
		0.897 0.672 1316 Anaerotruncus colihominis (T); 14565; AJ315980	
	620,75	0.953 0.804 1298 Eubacterium siraeum (T); L34625	0.980 0.954 1302 uncultured bacterium; RL199_aaj43c03; DQ793399
	723,75	0.947 0.795 1326 Clostridium sp. BI-114 (T); type strain:BI 114=CCUG 47106=DSM 15176; AJ518869	0.979 0.951 1302 uncultured bacterium; RL199_aaj41h12; DQ793301
	790,93	0.975 0.962 1326 Clostridium sp. BI-114 (T); type strain:BI 114=CCUG 47106=DSM 15176; AJ518869	0.980 0.973 1298 uncultured bacterium; RL305aal87h04; DQ799577

7.3.2. Cluster analysis and Jackknife

Cluster analysis and Jackknife test of PCR-DGGE fingerprinting showed a tendency for clustering for omnivores whereas vegetarians and elderly could not be grouped (Figures 11 and Table 6).

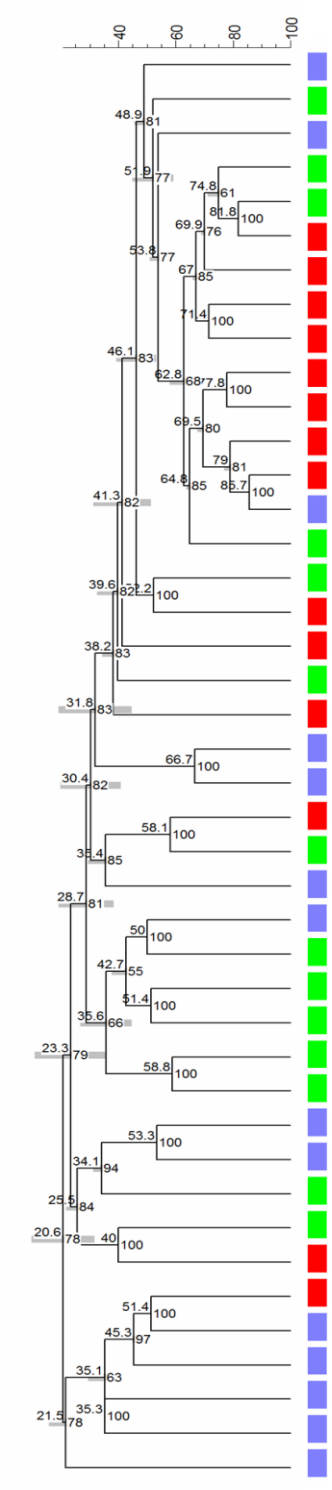

Figure 11 Cluster analysis of *Clostridium* Cl IV - DGGE-fingerprints (Dice coefficient).

Error flags, similarity values and cophenetic correlations are shown in the tree. Vegetarians green, omnivores red, elderly blue

Table 6 Jackknife (overall 54,76 %) of *Clostridium* cluster IV-DGGE fingerprints.

	Vegetarians	Omnivores	Elderly
Vegetarians	28.57	0.00	7.14
Omnivores	57.14	100.00	57.14
Elderly	14.29	0.00	35.71

7.3.3. Principal component analysis (PCA)

The highly diverse datasets of all bacterial groups were subjected to principal component analysis (PCA) which extracts underlying components within the dataset, separating samples according to their variance. This procedure resulted in a separation of omnivores and vegetarians according to their *Clostridium* cluster IV fingerprint and PCA also indicated a separation of young omnivores and elderly (Figure 12).

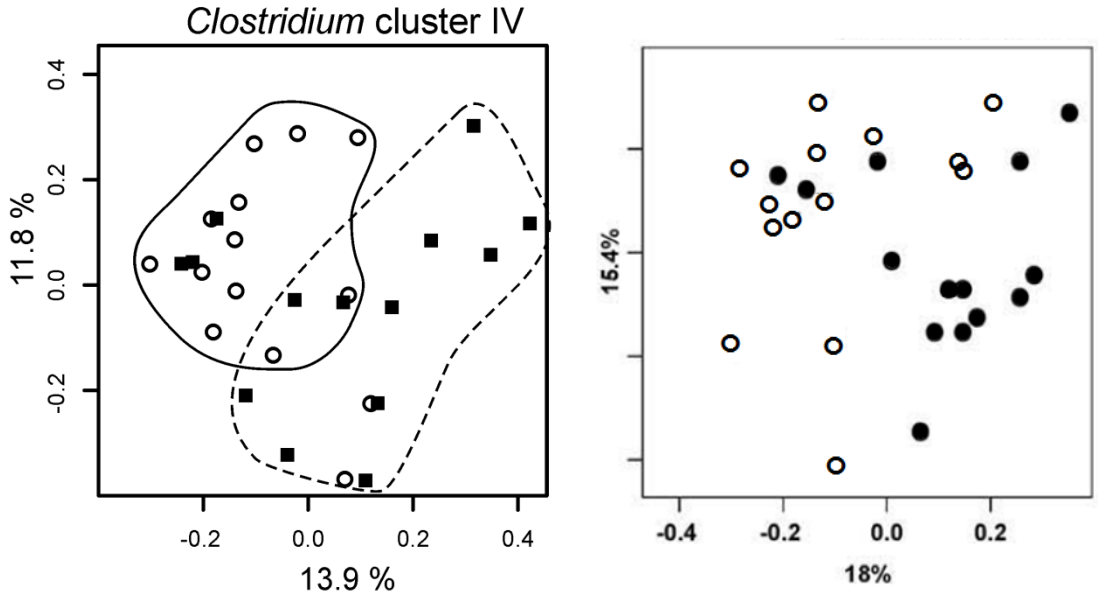

Figure 12 PCA of DGGE fingerprints of 16S rRNA coding regions of *Clostridium* cluster IV in faecal samples.

○, omnivores; ■, vegetarians; ●, elderly

7.3.4. Diversity indices

Both diversity indices (shannon and simpson) were significantly ($p < 0.02$) lower for aged citizens than for the young (Figure 13).

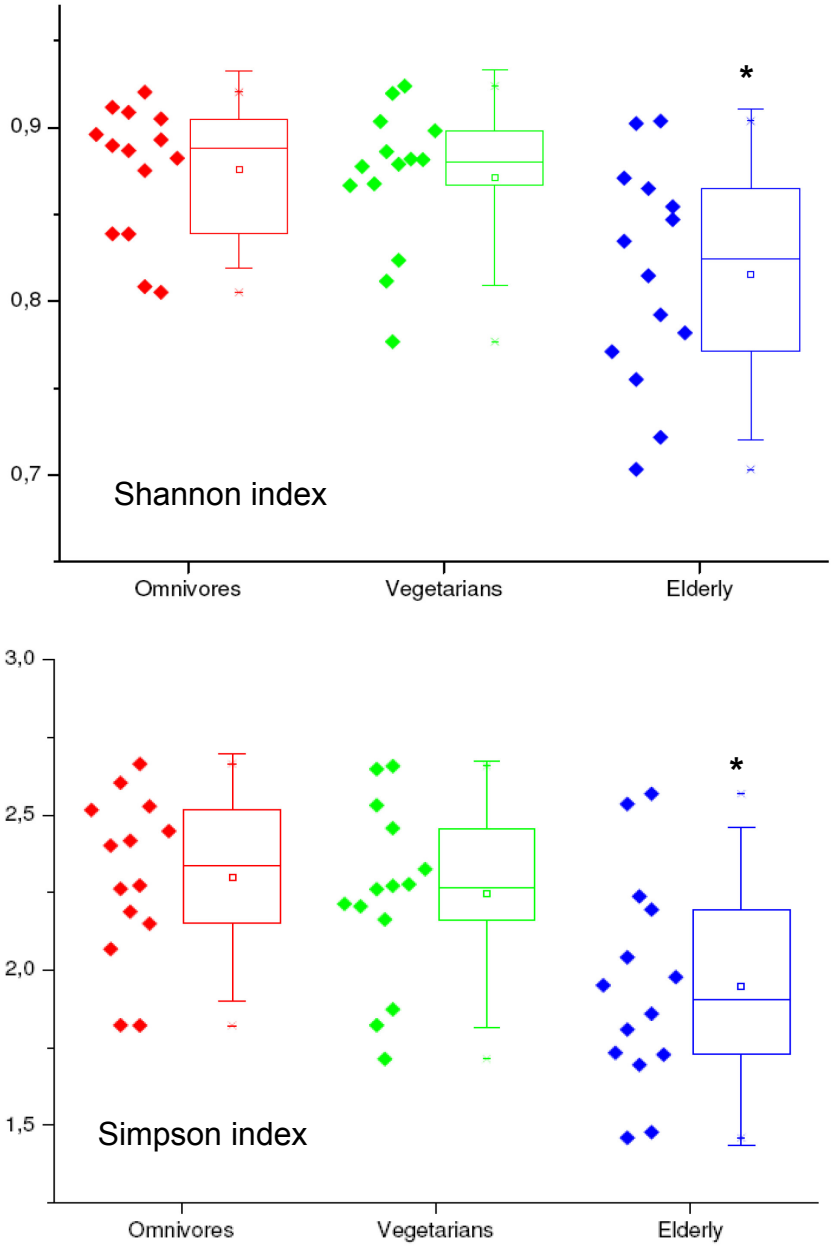

Figure 13 Diversity indices derived from DGGE fingerprinting of 16S rRNA coding regions.

red, Omnivores; green, Vegetarians; blue, Elderly; *, $p<0.02$.

8. Discussion

8.1. Methods

8.1.1. Study population

8.1.1.1 Diet

Vegetarianism is in some aspects heterogenous, because there are different forms of vegetarian diets like vegan or some who eat fish and not every vegetarian has a healthier food intake with higher amounts of vegetables and fruits, some of them prefer sweets. However, it is very hard to get trial subjects with a specified diet over months and years. To overcome this problem we used a questionnaire and defined the following facts which are guilty for the group of vegetarians: no intake of meat, higher intake of vegetables and fruits and those are the differences to the control group of omnivores.

8.1.1.2. Elderly

The majority of the group of the institutionalized elderly is bed ridden and received supplements with soluble fiber (Benfiber, Novartis). Those are two important points, which may influence the fecal microbiota. However, the prebiotic intervention alone was apparently not able to antagonize ageing-related changes in the microbiota [ZWIELEHNER et al., 2009].

8.1.2. Faecal samples

We used faecal samples in this study and faecal samples represent rather the microbiota of the colonic as of the whole gut. In the colon lives the greatest part of the gut microbiota, but also in the other parts huge masses of microorganisms exist. Therefore we did not get information about the microbial content of the small intestine where considerable microbe-host biological interaction occurs [TURNBAUGH et al., 2007]. Another very important point is that colonic microbes are probably not in direct contact with the mucosa and therefore no significant difference was found between colonic biopsies and

feces [MUYZER, 1999]. Therefore analyses of faecal samples have been shown to reflect adequate important changes [STEPHEN et al., 1987]. Besides them the samples are easy to collect and you do not need ethical issues.

8.1.3. Quantisation by TaqMan-PCR

Quantitative real time PCR is a very well established and sensitive method for detecting quantitative changes in gene expression as well as in quantisation of 16S rRNA coding regions of bacterial subgroups in faecal samples.

The difficulty lays in the comparison of different samples because the composition of feces is very inconsistent. That means, even you take the same weight of each sample the fiber, water and microorganism content is different. To overcome these problem bacterial subgroups were quantified as percentage of the total bacterial DNA and then compared with the other samples. In other words, we used the primers which detect nearly all bacteria as an intern standard or like a normaliser gene in the quantisation of gene expression.

8.1.4. PCR-DGGE

PCR-DGGE is a well-established method in molecular biology for studying complex ecosystems like microbial communities. Furthermore, this method allows identifying community members by sequencing excised bands. However, DGGE has also his limitations like the detection of heteroduplex molecules, molecules produced by different rRNA operons of the same organism or the limited sensitivity of detection of rare community members. Especially in the group of elderly the reduced diversity may occur due to the last point. Nevertheless, analyzing software has improved and is able to reduce mistakes. Thus in sum, PCR-DGGE is a reliable, reproducible, inexpensive, rapid and an appropriate method for detecting microbial diversity in different study groups [SILK et al., 2001].

8.2. Results

The human intestinal microbiota is a large, active und very complex ecosystem in our gut with marked influences on our metabolism and therefore important in health and disease. We used qPCR and PCR-DGGE for the quantitative and qualitative analysis of Clostridium cluster IV in vegetarians, omnivores and elderly.

8.2.1. Vegeterianism

Vegetarianism has been frequently associated with a decreased risk for different diseases. Therefore, we wanted to explore how vegetarian diet influences the human intestinal microbiota.

We found in vegetarians 19 % higher counts of bacterial DNA than in omnivores. Stephen et al showed in his study that a decreased transit time of stool is significant correlated to a higher bacterial cell mass [STEPHEN et al., 1987]. We could confirm with our questionnaire that vegetarians have a higher dietary fibre intake, which leads to a decreased transit time [SILK et al., 2001]. Those facts as well as pre-biotic consequences might contribute to the observation of higher counts of bacterial DNA in vegetarians.

Clostridium cluster IV also play an important role in the hydrolysis and fermentation of endogenous mucins and probably dietary protein, as well as in the conversion of bile acids and the production of toxins [NARUSHIMA et al., 2000]. Mueller et al. detected in a cross-sectional study the highest levels of the *Clostridium* cluster IV in their Swedish study population, whose dietary habits were characterized by a high consumption of fish and meat [MUELLER et al., 2006]. Therefore, we suppose that higher meat consumptions may increase the abundance of the *Clostridium* cluster IV in the gut microbiota. A higher prevalence of this subgroup was discussed to be associated with obesity and cancer [LI et al., 2008; SCANLAN et al., 2008].

PCA of PCR-DGGE fingerprinting of *Clostridium* cluster IV indicated a separation of vegetarians and omnivores in our study. Separation of *Clostridium* cluster IV might be due to the observation that two sequences identified as *Faecalibacterium* sp. and *Ruminococcaceae* sp. were more prevalent in omnivores than in vegetarians, probably due to effects of higher meat consumption as discussed for Clostridium cluster IV in general.

Despite of enormous interindividual deviations we observed a tendency for smaller abundances of *Clostridium* cluster IV in vegetarians. This might suggest a smaller capacity for energy harvest from food in vegetarians. Higher abundances of fibrolytic *Bacteroides* were found and might compensate for reductions in *Clostridium* cluster IV [LISZT et al., 2009].

8.2.2. Ageing

Our results indicate reductions of relative numbers of members of *Clostridium* cluster IV in the group of elderly. Mueller et al reported a significant reduction of *Faecalibacterium prausnitzii*, a prominent member of the Clostridium cluster IV, in Italian and Swedish elderly, while in German and French elderly this observation were not found [MUELLER et al., 2006]. These changes seem to be country-dependent or rather to depend on the characteristic colonization pattern of each country. Furthermore, the interplay with bacterial subgroups influence an in- or decrease in this group. A Chinese study by Li et al. also detected by using the new method PCR-ligase detection reaction (PCR-LDR) a significant reduction of *Clostridium leptum* group in elderly [LI et al., 2009]. Those studies could confirm our results.

Furthermore, we detect a significant less diversity in the PCR-DGGE fingerprints in geriatric patients, this could may have been observed because of the general lesser amount of bacterial DNA in the samples of this group or a hint for inadequate growing conditions in the gut of elderly.

Reduced numbers of bacteria in faecal content of elderly are a sign of the physiological alterations related with ageing, such as prolonged colonic transit

time and reduced dietary energy requirement and food uptake. Reductions of important sub-populations such as *Clostridium* cluster IV might result in reduced formation of SCFAs, changed epithelial cell maturation, maintenance and altered barrier function of the gut epithelium in elderly volunteers. Those alterations in the GIT microbiota have previously been linked to impaired immune functions common in individuals of advanced age and may result in a greater susceptibility to disease. [ZWIELEHNER et al., 2009]

Improved analytical concepts for the characterization of the microbiota of consumers and patients might become important as a rationale for individualized therapeutic intervention. For example probiotic, prebiotic and synbiotic supplementation is a promising concept in restoring impaired functions or enhancing specific desirable functions of the microbiota. Even if some studies at the moment are contradictory, probiotic and rather prebiotic intervention are promising therapeutics. Further investigations are mandatory to gain a deeper insight into the relationships between phylogenetic information and metabolic activities.

9. Conclusion

This diploma thesis could show that vegetarian diet as well as ageing affects the intestinal microbiota, especially in decreasing the amount and changing the diversity of *Clostridium* cluster IV. It remains to be determined how these shifts might affect host metabolism and disease risks and how this ecosystem can be influenced by treatment with pro- or prebiotics.

10. Summary

The human body especially the human intestinal tract harbors a large, active and very complex community of microbes with important influence on our health maintenance [HOOPER and GORDON, 2001]. Furthermore, in different studies the microbiota especially *Clostridium* cluster IV a predominant cluster of the faecal microbiota is discussed to be associated with diseases like inflammatory bowel diseases [SOKOL et al., 2006], cancer [SCANLAN et al., 2008] and obesity [TURNBAUGH et al., 2009]. Therefore, it is of interest if this cluster can be influenced by diet like vegetarianism and if it changes in elderly. The aim of this thesis was to investigate the quantitative and qualitative changes of *Clostridium* cluster IV in faecal microbiota associated with vegetarian diet and ageing.

Bacterial abundances were measured in faecal samples of 15 elderly (aged 86±8 years, BMI 21.75 ± 5.08), 15 vegetarians (aged 23.2 ± 2.4 years, BMI 20.44 ± 1.77) and 14 omnivores (aged 24±2.5 years, BMI 22.68 ± 3.41) using quantitative real time PCR (qPCR). Diversity was assessed with PCR-DGGE fingerprinting, PCA (principal components analysis) and Shannon and Simpson diversity index. Interesting sequences have been sequenced and analysed in RDP 9.58 (ribosomal database project, http://rdp.cme.msu.edu/).

Vegetarians showed 19 % higher whereby elderly showed 26 % lesser counts of bacterial DNA than omnivores. However, these differences were not significant due to high interindividual deviations. The mean proportion of *Clostridium* cluster IV in stool samples of vegetarians was 31.86 ± 17.00 %, in omnivores 36.64 ± 14.22 % and in elderly 27.20 ± 14.63 %. The three vegans did not stand out in the group of vegetarians. Cluster analysis and Jackknife test of PCR-DGGE fingerprinting showed a tendency for clustering for omnivores whereas vegetarians and elderly could not be grouped. PCA suggested a separation of omnivores in both separated PCA analysis of vegetarians to omnivores and elderly to omnivores. Shannon and Simpson diversity indices showed a significant decrease in diversity of elderly compared to both young

groups ($p<0.02$). Two bands (one 96.7 % similar to *Faecalibacterium prausnitzii*^{T}, the other an uncultured bacterium DQ793301with 97.9 % similarity) of the PCR-DGGE-fingerprints were more prevalent in omnivores than in vegetarians and elderly. One band with 97.5 % similarity to Clostridium sp. BI-114^T) was significantly more prevalent in omnivores than in elderly and another band (uncultured bacterium DQ793399 with 98.0 % similarity) was significantly more prevalent in elderly than in vegetarians and for a tendency in omnivores.

This diploma thesis could show that vegetarian diet as well as ageing affects the intestinal microbiota, especially in decreasing the amount and changing the diversity of *Clostridium* cluster IV. It remains to be determined how these shifts might affect host metabolism and disease risks and how this ecosystem can be influenced by treatment with pro- or prebiotics.

11. Zusammenfassung

Der menschliche Körper speziell der Intestinaltrakt enthält eine große, aktive und sehr komplexe Gemeinschaft von Mikroorganismen die eine entscheidende Rolle für die Gesunderhaltung unseres Körpers spielen. *Clostridium* cluster IV einer der vorherrschenden Cluster im Darmtrakt wurde in verschiedenen Studien im Zusammenhang mit Erkrankungen wie entzündliche Darmerkrankungen [SOKOL et al., 2006], Colonkrebs [SCANLAN et al., 2008] und krankhaftem Übergewicht [TURNBAUGH et al., 2009] diskutiert. Daher ist es von besonderem Interesse ob dieser Cluster nun durch Diät im speziellen durch eine vegetarische Diät bzw. im Alter verändert wird. Das Ziel dieser Diplomarbeit war es die qualitativen und quantitativen Veränderungen von *Clostridium* cluster IV im fäkalen Mikrobiom von Vegetariern und in Alten zu untersuchen.

Die Menge an Gesamtbakterien und der bakteriellen Subgruppe wurde in Stuhlproben von 15 Geriatriepatienten (86±8 Jahren, BMI 21,75 ± 5,08), 15 Vegetariern (23,2 ± 2,4 Jahren, BMI 20,44 ± 1,77) und 14 Omnivoren/Mischköstern (24±2,5 Jahren, BMI 22,68 ± 3,41) mit Hilfe von quantitativer real time polymerase chain reaction (qPCR) gemessen. Die Diversität mit PCR-denaturierender Gradienten Gelelektrophorese (PCR-DGGE). Für die statistische Analyse der Datenmenge verwendeten wir die Hauptkomponentenanalyse (PCA, principal component analysis), Shannon und Simpson Diversität index. Banden von besonderem Interesse wurden sequenziert und mit Hilfe der Datenbank des RDP 9.58 (ribosomal database project, http://rdp.cme.msu.edu/) analysiert.

In Vegetariern zeigten sich 19 % höhere und in den Geriatriepatienten 26 % geringere Werte von bakterieller DNA als in Mischköstern. Diese Unterschiede waren jedoch aufgrund hoher interindividueller Abweichungen nicht signifikant. Die Mittelwerte der prozentuellen Anteile von *Clostridium* cluster IV zu Gesamtbakterien in den Stuhlproben waren bei Vegetariern 31,86 ± 17,00 %, in Omnivoren 36,64 ± 14,22 % und in den Alten 27,20 ± 14,63 %. Die 3 Veganen

waren nicht auffällig in der Gruppe der Vegetarier. Die Clusteranalyse und der Jackknifetest der PCR-DGGE fingerprints zeigten eine Tendenz für die Clusterbildung der Omnivoren, während bei Vegetariern und Alten keine Gruppierung ersichtlich war. Die Hauptkpomponentenanalyse zeigte in beiden plots Omnivoren zu Vegetariern und Omnivore zu Alten eine Trennung der jeweiligen Gruppen. Shannon und Simpson Diversitätsindex zeigten eine signifikante Abnahme der Diversität in der Gruppe der Geriatriepatienten im Vergleich zu den beiden jungen Gruppen ($p<0.02$).

2 Banden (eine mit 96.7 % Ähnlichkeit zu *Faecalibacterium prausnitzii*^{T}, die andere mit dem unkultivierten Bakterium DQ793301 97.9 % Ähnlichkeit) aus den PCR-DGGE-fingerprints kamen signifikant ($p<0,05$) häufiger in Omnivoren als in Vegetariern und Alten vor, während die Bande mit 97.5 %iger Ähnlichkeit zu *Clostridium* sp. BI-114^T nur signifikant seltener in Alten vorkam. Allerdings eine Bande (unkultiviertes Bakterium DQ793399 with 98.0 % Ähnlichkeit) kam signifikant häufiger in Alten als in Vegetariern und tendenziell häufiger in Omnivoren.

Diese Diplomarbeit zeigt, dass sowohl eine vegetarische Diät als auch das Alter Einfluss auf unsere Darmbakterien haben, im speziellen durch Dezimierung und Veränderung der Diversität von *Clostridium* cluster IV. Es bleibt jedoch weiterhin die Frage offen, wie diese Veränderungen möglicherweise unseren Metabolismus bzw. unser Erkrankungsrisiko beeinflussen und wie dieses Ökosystem durch Behandlung mit z.B. Pro- und Präbiotiker verändert werden kann.

12. References

Position of the american dietetic association and dietitians of canada: Vegetarian diets. J Am Diet Assoc 2003;103:748-65.

ALM, J S, SWARTZ, J, BJORKSTEN, B, ENGSTRAND, L, ENGSTROM, J, KUHN, I, LILJA, G, MOLLBY, R, NORIN, E, PERSHAGEN, G, REINDERS, C, WREIBER, K and SCHEYNIUS, A. An anthroposophic lifestyle and intestinal microflora in infancy. Pediatr Allergy Immunol 2002;13:402-11.

BARTOSCH, S, FITE, A, MACFARLANE, G T and MCMURDO, M E. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time pcr and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol 2004;70:3575-81.

CANI, P D, NEYRINCK, A M, FAVA, F, KNAUF, C, BURCELIN, R G, TUOHY, K M, GIBSON, G R and DELZENNE, N M. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia 2007;50:2374-83.

COLLINS, M D, LAWSON, P A, WILLEMS, A, CORDOBA, J J, FERNANDEZ-GARAYZABAL, J, GARCIA, P, CAI, J, HIPPE, H and FARROW, J A. The phylogeny of the genus clostridium: Proposal of five new genera and eleven new species combinations. Int J Syst Bacteriol 1994;44:812-26.

CUMMINGS, J H, BEATTY, E R, KINGMAN, S M, BINGHAM, S A and ENGLYST, H N. Digestion and physiological properties of resistant starch in the human large bowel. Br J Nutr 1996;75:733-47.

DETHLEFSEN, L, ECKBURG, P B, BIK, E M and RELMAN, D A. Assembly of the human intestinal microbiota. Trends Ecol Evol 2006;21:517-23.

DICKSVED, J, FLOISTRUP, H, BERGSTROM, A, ROSENQUIST, M, PERSHAGEN, G, SCHEYNIUS, A, ROOS, S, ALM, J S, ENGSTRAND, L, BRAUN-FAHRLANDER, C, VON MUTIUS, E and JANSSON, J K. Molecular fingerprinting of the fecal microbiota of children raised according to different lifestyles. Applied and Environmental Microbiology 2007;73:2284-2289.

DUNCAN, S H, HOLD, G L, HARMSEN, H J, STEWART, C S and FLINT, H J. Growth requirements and fermentation products of fusobacterium prausnitzii, and a proposal to reclassify it as faecalibacterium prausnitzii gen. Nov., comb. Nov. Int J Syst Evol Microbiol 2002;52:2141-6.

ECKBURG, P B, BIK, E M, BERNSTEIN, C N, PURDOM, E, DETHLEFSEN, L, SARGENT, M, GILL, S R, NELSON, K E and RELMAN, D A. Diversity of the human intestinal microbial flora. Science (New York, N.Y.) 2005;308:1635-1638.

EDWARDS, U, ROGALL, T, BLOCKER, H, EMDE, M and BOTTGER, E C. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16s ribosomal rna. Nucleic Acids Res 1989;17:7843-53.

EWASCHUK, J B and DIELEMAN, L A. Probiotics and prebiotics in chronic inflammatory bowel diseases. World J Gastroenterol 2006;12:5941-50.
FAMULARO, G, DE SIMONE, C, PANDEY, V, SAHU, A R and MINISOLA, G. Probiotic lactobacilli: An innovative tool to correct the malabsorption syndrome of vegetarians? Medical hypotheses 2005;65:1132-1135.
FANARO, S, CHIERICI, R, GUERRINI, P and VIGI, V. Intestinal microflora in early infancy: Composition and development. Acta Paediatr Suppl 2003;91:48-55.
FORSYTHE, P, SUDO, N, DINAN, T, TAYLOR, V H and BIENENSTOCK, J. Mood and gut feelings. Brain Behav Immun 2009.
FRANK, D N, ST AMAND, A L, FELDMAN, R A, BOEDEKER, E C, HARPAZ, N and PACE, N R. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A 2007;104:13780-5.
GILL, S R, POP, M, DEBOY, R T, ECKBURG, P B, TURNBAUGH, P J, SAMUEL, B S, GORDON, J I, RELMAN, D A, FRASER-LIGGETT, C M and NELSON, K E. Metagenomic analysis of the human distal gut microbiome. Science (New York, N.Y.) 2006;312:1355-1359.
GUIGOZ, Y, DORE, J and SCHIFFRIN, E J. The inflammatory status of old age can be nurtured from the intestinal environment. Curr Opin Clin Nutr Metab Care 2008;11:13-20.
HAYASHI, H, SAKAMOTO, M and BENNO, Y. Fecal microbial diversity in a strict vegetarian as determined by molecular analysis and cultivation. Microbiol Immunol 2002;46:819-31.
HOOPER, L V and GORDON, J I. Commensal host-bacterial relationships in the gut. Science 2001;292:1115-8.
KIRJAVAINEN, P V, APOSTOLOU, E, ARVOLA, T, SALMINEN, S J, GIBSON, G R and ISOLAURI, E. Characterizing the composition of intestinal microflora as a prospective treatment target in infant allergic disease. FEMS Immunol Med Microbiol 2001;32:1-7.
LAY, C, SUTREN, M, ROCHET, V, SAUNIER, K, DORE, J and RIGOTTIER-GOIS, L. Design and validation of 16s rrna probes to enumerate members of the clostridium leptum subgroup in human faecal microbiota. Environmental microbiology 2005;7:933-946.
LEY, R E, HAMADY, M, LOZUPONE, C, TURNBAUGH, P J, RAMEY, R R, BIRCHER, J S, SCHLEGEL, M L, TUCKER, T A, SCHRENZEL, M D, KNIGHT, R and GORDON, J I. Evolution of mammals and their gut microbes. Science 2008;320:1647-51.
LEY, R E, PETERSON, D A and GORDON, J I. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 2006;124:837-48.
LEY, R E, TURNBAUGH, P J, KLEIN, S and GORDON, J I. Microbial ecology: Human gut microbes associated with obesity. Nature 2006;444:1022-3.
LI, K, CHEN, B, ZHOU, Y, HUANG, R, LIANG, Y, WANG, Q, XIAO, Z and XIAO, J. Multiplex quantification of 16s rdna of predominant bacteria group within human fecal samples by polymerase chain reaction--ligase detection reaction (pcr-ldr). J Microbiol Methods 2009;76:289-94.

LI, M, WANG, B, ZHANG, M, RANTALAINEN, M, WANG, S, ZHOU, H, ZHANG, Y, SHEN, J, PANG, X, ZHANG, M, WEI, H, CHEN, Y, LU, H, ZUO, J, SU, M, QIU, Y, JIA, W, XIAO, C, SMITH, L M, YANG, S, HOLMES, E, TANG, H, ZHAO, G, NICHOLSON, J K, LI, L and ZHAO, L. Symbiotic gut microbes modulate human metabolic phenotypes. Proc Natl Acad Sci U S A 2008;105:2117-22.

LIM, C C, FERGUSON, L R and TANNOCK, G W. Dietary fibres as "Prebiotics": Implications for colorectal cancer. Mol.Nutr.Food Res. 2005;49:609-619.

LIM, C C, FERGUSON, L R and TANNOCK, G W. Dietary fibres as "Prebiotics": Implications for colorectal cancer. Mol Nutr Food Res 2005;49:609-19.

LISZT, K, ZWIELEHNER, J, HANDSCHUR, M, HIPPE, B, THALER, R and HASLBERGER, A G. Characterization of bacteria, clostridia and bacteroides in faeces of vegetarians using qpcr and pcr-dgge fingerprinting. Ann Nutr Metab 2009;54:253-257.

LOUIS, P, SCOTT, K P, DUNCAN, S H and FLINT, H J. Understanding the effects of diet on bacterial metabolism in the large intestine. J Appl Microbiol 2007;102:1197-208.

MANICHANH, C, RIGOTTIER-GOIS, L, BONNAUD, E, GLOUX, K, PELLETIER, E, FRANGEUL, L, NALIN, R, JARRIN, C, CHARDON, P, MARTEAU, P, ROCA, J and DORE, J. Reduced diversity of faecal microbiota in crohn's disease revealed by a metagenomic approach. Gut 2006;55:205-211.

MATSUKI, T, WATANABE, K, FUJIMOTO, J, TAKADA, T and TANAKA, R. Use of 16s rrna gene-targeted group-specific primers for real-time pcr analysis of predominant bacteria in human feces. Applied and Environmental Microbiology 2004;70:7220-7228.

MORLEY, J E. The aging gut: Physiology. Clin Geriatr Med 2007;23:757-67, v-vi.

MUELLER, S, SAUNIER, K, HANISCH, C, NORIN, E, ALM, L, MIDTVEDT, T, CRESCI, A, SILVI, S, ORPIANESI, C, VERDENELLI, M C, CLAVEL, T, KOEBNICK, C, ZUNFT, H J, DORE, J and BLAUT, M. Differences in fecal microbiota in different european study populations in relation to age, gender, and country: A cross-sectional study. Appl Environ Microbiol 2006;72:1027-33.

MUELLER, S, SAUNIER, K, HANISCH, C, NORIN, E, ALM, L, MIDTVEDT, T, CRESCI, A, SILVI, S, ORPIANESI, C, VERDENELLI, M C, CLAVEL, T, KOEBNICK, C, ZUNFT, H J, DORE, J and BLAUT, M. Differences in fecal microbiota in different european study populations in relation to age, gender, and country: A cross-sectional study. Applied and Environmental Microbiology 2006;72:1027-1033.

MUYZER, G. Dgge/tgge a method for identifying genes from natural ecosystems. Current opinion in microbiology 1999;2:317-322.

MUYZER, G, DE WAAL, E C and UITTERLINDEN, A G. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16s rrna. Appl Environ Microbiol 1993;59:695-700.

NARUSHIMA, S, ITO, K, KURUMA, K and UCHIDA, K. Composition of cecal bile acids in ex-germfree mice inoculated with human intestinal bacteria. Lipids 2000;35:639-44.
PENDERS, J, STOBBERINGH, E E, VAN DEN BRANDT, P A and THIJS, C. The role of the intestinal microbiota in the development of atopic disorders. Allergy 2007;62:1223-36.
SARTOR, R B. Microbial influences in inflammatory bowel diseases. Gastroenterology 2008;134:577-94.
SCANLAN, P D, SHANAHAN, F, CLUNE, Y, COLLINS, J K, O'SULLIVAN, G C, O'RIORDAN, M, HOLMES, E, WANG, Y and MARCHESI, J R. Culture-independent analysis of the gut microbiota in colorectal cancer and polyposis. Environ Microbiol 2008;10:789-98.
SILK, D B, WALTERS, E R, DUNCAN, H D and GREEN, C J. The effect of a polymeric enteral formula supplemented with a mixture of six fibres on normal human bowel function and colonic motility. Clin Nutr 2001;20:49-58.
SOKOL, H, PIGNEUR, B, WATTERLOT, L, LAKHDARI, O, BERMUDEZ-HUMARAN, L G, GRATADOUX, J J, BLUGEON, S, BRIDONNEAU, C, FURET, J P, CORTHIER, G, GRANGETTE, C, VASQUEZ, N, POCHART, P, TRUGNAN, G, THOMAS, G, BLOTTIERE, H M, DORE, J, MARTEAU, P, SEKSIK, P and LANGELLA, P. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of crohn disease patients. Proc Natl Acad Sci U S A 2008;105:16731-6.
SOKOL, H, SEKSIK, P, RIGOTTIER-GOIS, L, LAY, C, LEPAGE, P, PODGLAJEN, I, MARTEAU, P and DORE, J. Specificities of the fecal microbiota in inflammatory bowel disease. Inflamm Bowel Dis 2006;12:106-11.
SONG, Y, LIU, C and FINEGOLD, S M. Real-time pcr quantitation of clostridia in feces of autistic children. Appl Environ Microbiol 2004;70:6459-65.
STEPHEN, A M, WIGGINS, H S and CUMMINGS, J H. Effect of changing transit time on colonic microbial metabolism in man. Gut 1987;28:601-9.
STEWART, J A, CHADWICK, V S and MURRAY, A. Investigations into the influence of host genetics on the predominant eubacteria in the faecal microflora of children. J Med Microbiol 2005;54:1239-42.
SUAU, A, ROCHET, V, SGHIR, A, GRAMET, G, BREWAEYS, S, SUTREN, M, RIGOTTIER-GOIS, L and DORE, J. Fusobacterium prausnitzii and related species represent a dominant group within the human fecal flora. Syst Appl Microbiol 2001;24:139-45.
TURNBAUGH, P J, HAMADY, M, YATSUNENKO, T, CANTAREL, B L, DUNCAN, A, LEY, R E, SOGIN, M L, JONES, W J, ROE, B A, AFFOURTIT, J P, EGHOLM, M, HENRISSAT, B, HEATH, A C, KNIGHT, R and GORDON, J I. A core gut microbiome in obese and lean twins. Nature 2009;457:480-4.
TURNBAUGH, P J, LEY, R E, HAMADY, M, FRASER-LIGGETT, C M, KNIGHT, R and GORDON, J I. The human microbiome project. Nature 2007;449:804-10.

VAEL, C and DESAGER, K. The importance of the development of the intestinal microbiota in infancy. Curr Opin Pediatr 2009.

VAN DER WAAIJ, L A, HARMSEN, H J, MADJIPOUR, M, KROESE, F G, ZWIERS, M, VAN DULLEMEN, H M, DE BOER, N K, WELLING, G W and JANSEN, P L. Bacterial population analysis of human colon and terminal ileum biopsies with 16s rrna-based fluorescent probes: Commensal bacteria live in suspension and have no direct contact with epithelial cells. Inflamm Bowel Dis 2005;11:865-71.

VINNARI, M, MONTONEN, J, HARKANEN, T and MANNISTO, S. Identifying vegetarians and their food consumption according to self-identification and operationalized definition in finland. Public Health Nutr 2008:1-8.

WIKOFF, W R, ANFORA, A T, LIU, J, SCHULTZ, P G, LESLEY, S A, PETERS, E C and SIUZDAK, G. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci U S A 2009;106:3698-703.

WOODMANSEY, E J. Intestinal bacteria and ageing. J Appl Microbiol 2007;102:1178-86.

WOODMANSEY, E J, MCMURDO, M E, MACFARLANE, G T and MACFARLANE, S. Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. Appl Environ Microbiol 2004;70:6113-22.

YOUNGSEOB, Y, CHANGSOO, L, JAAI, K, SEOKHWAN H. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Wiley InterScience 2005.

YU, Y, LEE, C, KIM, J and HWANG, S. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol Bioeng 2005;89:670-9.

ZWIELEHNER, J, LISZT, K, HANDSCHUR, M, LASSL, C, LAPIN, A and HASLBERGER, A G. Combined pcr-dgge fingerprinting and quantitative-pcr indicates shifts in fecal population sizes and diversity of bacteroides, bifidobacteria and clostridium cluster iv in institutionalized elderly. Exp Gerontol 2009;44:440-6.

13. Published research data

13.1. Published research article - Characterization of *Bacteria*, *Clostridia*, *Bacteroides* in faeces of vegetarians using qPCR and PCR-DGGE fingerprinting

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Characterization of Bacteria, Clostridia and *Bacteroides* in Faeces of Vegetarians Using qPCR and PCR-DGGE Fingerprinting

Kathrin Liszt Jutta Zwielehner Michael Handschur Berit Hippe Roman Thaler
Alexander G. Haslberger

Department of Nutritional Sciences, University of Vienna, Vienna, Austria

Key Words
Microbiota · PCR-DGGE · qPCR · *Clostridium* cluster IV · *Bacteroides* · *Bifidobacterium* · Faeces · Vegetarians

Abstract
Background/Aims: This study aimed to investigate the quantitative and qualitative changes of bacteria, *Bacteroides*, *Bifidobacterium* and *Clostridium* cluster IV in faecal microbiota associated with a vegetarian diet. ***Methods:*** Bacterial abundances were measured in faecal samples of 15 vegetarians and 14 omnivores using quantitative PCR. Diversity was assessed with PCR-DGGE fingerprinting, principal component analysis (PCA) and Shannon diversity index. ***Results:*** Vegetarians had a 12% higher abundance of bacterial DNA than omnivores, a tendency for less *Clostridium* cluster IV (31.86 ± 17.00%; 36.64 ± 14.22%) and higher abundance of *Bacteroides* (23.93 ± 10.35%; 21.26 ± 8.05%), which were not significant due to high interindividual variations. PCA suggested a grouping of bacteria and members of *Clostridium* cluster IV. Two bands appeared significantly more frequently in omnivores than in vegetarians ($p < 0.005$ and $p < 0.022$). One was identified as *Faecalibacterium* sp. and the other was 97.9% similar to the uncultured gut bacterium DQ793301. ***Conclusions:*** A vegetarian diet affects the intestinal microbiota, especially by decreasing the amount and changing the diversity of *Clostridium* cluster IV. It remains to be determined how these shifts might affect the host metabolism and disease risks.

Introduction

Vegetarianism has been associated with a decreased risk for diseases such as heart diseases and various cancers and has been linked to a lower BMI and an overall decline in mortality [1]. Different lifestyle factors and diets were shown to have a significant impact on the faecal microbiota [2, 3].

The highly diverse microbiota of the human gastrointestinal tract has been associated with colorectal cancer [4], inflammatory bowel diseases [5], obesity [6] and metabolic syndrome [7]. Two of the most predominant subpopulations in the human faecal microbiota are the *Clostridium* cluster IV (*Clostridium leptum* subgroup) and *Bacteroides* [8, 9]. Some members of these populations are fibrolytic and produce butyrate [10, 11]. The metabolic activities of these organisms have a significant influence on colonic health as butyrate is known as the major energy source for colonocytes [10] and as a possible epigenetic regulator of gene expression in colonic epithelium [12]. Epigenetic modifications have also been implicated in changes of gene expression associated with a vegetarian diet [13, 14].

Stimulation of *Bifidobacterium* has previously been shown after prebiotic intervention with inulin and fructo-oligosaccharides [15]. *Bifidobacterium* has been shown to be involved in the prevention of atopic disease [16] and the prevention of obesity and insulin resistance via enhanced barrier function of the gut epithelium [17].

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Univ.-Doz. Dr. Alexander Haslberger
Department of Nutritional Sciences
Althanstrasse 14
AT–1090 Vienna (Austria)
Tel. +43 142 775 4996, Fax +43 142 779 549, E-Mail alexander.haslberger@univie.ac.at

The previous molecular analysis of vegetarian gut microbiota of a single individual revealed that *Clostridium* cluster XIVa, *Clostridium* cluster IV, and *Clostridium* cluster XVIII were major components [18].

In the present study, we applied quantitative PCR (qPCR) and PCR-DGGE fingerprinting to investigate the dominant microbiota in 14 young omnivores and 15 vegetarians assessing *Clostridium* cluster IV, *Bacteroides* and *Bifidobacterium*. Analyses of faecal samples have been shown to reflect adequate important changes [19].

Material and Methods

Participants

Twenty-nine healthy young individuals, 15 vegetarians (aged 19–34 years, BMI 22.06 ± 3.82) and 14 omnivores (aged 21–31 years, BMI 21.02 ± 2.71), were compared. A questionnaire about dietary habits and health activities was given to all participants. Exclusion criteria were the use of antibiotics, chemotherapeutic treatment, and pre- and probiotics 3 months prior to sampling.

Sampling and DNA Extraction

Stool samples were immediately stored at –20°C after sampling. DNA was extracted using the DNA Stool Mini Kit (Qiagen) following the manufacturer's protocol with minor modifications and immediately stored at –20°C.

TaqMan qPCR

Bacterial 16S rRNA was quantified by TaqMan qPCR using previously published primers and probes [20–23]. TaqMan Probe Clept-P (AGG GTT GCG CTC GTT) was designed for *Clostridium* cluster IV in this study. DNA of *Bacteroides thetaiotaomicron* and *Bifidobacterium longum*, clone CL16 and one faecal sample were used to generate standard curves for comparison of PCR reaction efficiencies among different experiments and enumeration of all bacterial groups. Relative abundances of bacterial subgroups were calculated in relation to total 16S rRNA gene copies for each individual.

PCR-DGGE Fingerprinting

16S rRNA coding regions were amplified using group-specific primers [17, 24–29]. Reference markers containing fragments of 16S rRNA coding regions were loaded to each gel in triplicate to allow gel-to-gel comparison. DGGE gels were prepared as described previously [30].

Statistical Analysis

We analyzed qPCR results by F test and Student's t test. Food frequency data and interesting bands of DGGE fingerprints were analyzed based on χ^2 approximation as implemented in SPSS15. PCR-DGGE band comparison tables were created in GelComparII and analyzed with principal component analysis (PCA). The plots show the transformed data with the first two principal components as x- and y-axis.

Results

Dietary Aspects

Analysis of the participants' dietary habits indicated similar consumption patterns of liquids, alcohol, fruits, grains and milk products in both groups. Exercise levels were comparable. Omnivores stated significantly less frequent (χ^2 test; $p < 0.027$) consumption of vegetables than vegetarians. Three of the vegetarians assessed followed a vegan diet; all others followed a lacto-ovo vegetarian diet. Five vegetarians said that they eat fish a few times a year.

Relative Quantitation

Bifidobacterium, members of *Clostridium* cluster IV and *Bacteroides* were quantified as percentage of the total bacterial DNA. No cross-reactivity of group-specific primers and probes with nontarget strains could be detected. Test-retest variations were between 2.7 and 5.2%; values after relative quantification varied for less than 4%. Vegetarians showed 12% higher counts of bacterial DNA than omnivores but these differences were not significant due to high interindividual deviations. The mean proportion of *Clostridium* cluster IV in stool samples of vegetarians was 31.86 ± 17.00% and in omnivores 36.64 ± 14.22%. The mean percentage of *Bacteroides* in vegetarians was 23.93 ± 10.35% and in omnivores 21.26 ± 8.05%, while the mean proportion of *Bifidobacterium* in vegetarians (1.52 ± 1.29%) was unchanged compared to omnivores (1.59 ± 1.73%).

PCR-DGGE Quantitative Analysis

The highly diverse datasets of all bacterial groups were subjected to PCA, which extracts underlying components within the dataset, separating samples according to their variance. This procedure resulted in a separation of omnivores and vegetarians according to their *Clostridium* cluster IV fingerprint. Some grouping was also visible in the dominant bacteria dataset (fig. 1). The mean numbers of bands observed with a primer pair (341–518) targeting most bacteria were 20.1 ± 3.3 for omnivores and 18.07 ± 3.7 for vegetarians. The fingerprints of bacterial subgroups were similar for all participants. Band patterns of *Bacteroides* were composed of 9.5 ± 2.9 bands, *Clostridium* cluster IV of 12.75 ± 3.37 bands and 13.2 ± 3.1 bands related to *Bifidobacterium* spp. Shannon diversity indices based on the DGGE fingerprinting were similar for all bacterial groups. However, two bands (fig. 2) from *Clostridium* cluster IV were more prevalent in omnivores than in vegetarians (χ^2 test; $p < 0.005$; $p < 0.022$).

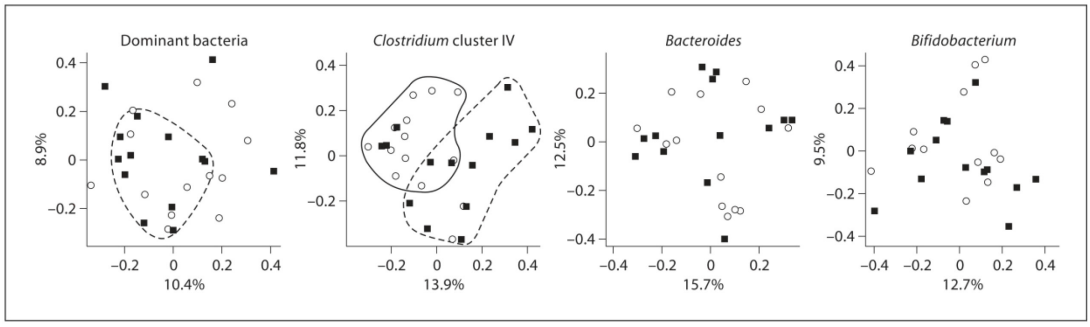

Fig. 1. PCA of DGGE fingerprints of 16S rRNA coding regions of dominant bacteria, *Clostridium* cluster IV, *Bacteroides* and *Bifidobacterium* in faecal samples. ○ = Omnivores; ■ = vegetarians.

Discussion

We found 12% higher counts of bacterial DNA in intestinal microbiota of vegetarians than in omnivores. A decreased transit time of stool correlating with higher bacterial cell mass due to higher dietary fibre intake [31, 32] as well as prebiotic consequences might contribute to this observation.

We could also see a tendency for higher abundance of *Bacteroides* and lower abundance of *Clostridium* cluster IV in faecal microbiota of vegetarians compared to omnivores. *Bacteroides* can utilize a wide variety of carbon sources, and they account for the majority of polysaccharide digestion in the large intestine [33, 34]. Nevertheless, members of *Clostridium* cluster IV have the same ability. We assume that dietary fibre encourages the growth of *Bacteroides*.

Clostridium cluster IV and *Bacteroides* also play an important role in the hydrolysis and fermentation of endogenous mucins and probably dietary protein, as well as in the conversion of bile acids and the production of toxins [34, 35]. In a cross-sectional study of different European populations, Mueller et al. [36] detected the highest levels of the *Clostridium* cluster IV in the Swedish study population, whose dietary habits were characterized by a high consumption of fish and meat. Therefore, we suppose that higher meat consumptions may increase the abundance of the *Clostridium* cluster IV in the gut microbiota. A higher prevalence of this subgroup was discussed to be associated with obesity and cancer [4, 6].

A previous study [18] involving only 1 patient discussed whether *Clostridium* cluster XIVa was affected by a vegetarian diet. We could not find noticeable dif-

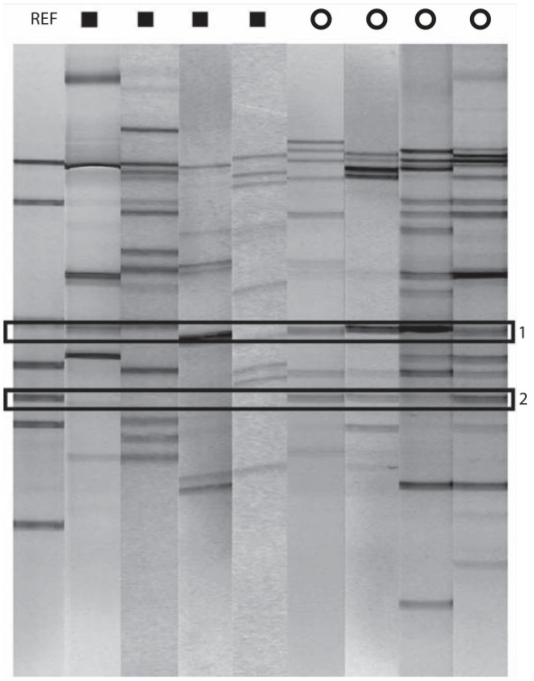

Fig. 2. PCR-DGGE band patterns of 16S rRNA coding regions of *Clostridium* cluster IV amplified with primer pair sg-Clept-F-GC/sg-Clept-R. Bands that were observed more frequently in omnivores than in vegetarians are indicated with a black box. 1 = *Clostridium* sp. BI-114 (similarity 94.7%) and uncultured bacterium DQ793301 (similarity 97.9%); 2 = 96.7% similar to *Faecalibacterium prausnitzii* and 99.5% similar to *F. prausnitzii* AJ270469. ○ = Omnivores; ■ = vegetarians; REF = reference lane.

ferences for this subgroup in our samples (data not shown).

PCR-DGGE fingerprinting of dominant bacteria and *Clostridium* cluster IV indicated a grouping of vegetarians and omnivores in our study. Clustering of *Clostridium* cluster IV fingerprints might be due to the observation that two sequences identified as *Faecalibacterium* sp. and Ruminococcaceae sp. were more prevalent in omnivores than in vegetarians, probably due to the effects of higher meat consumption as discussed for *Clostridium* cluster IV in general.

Despite enormous interindividual variations, we observed a tendency for smaller abundances of *Clostridium* cluster IV in vegetarians. This might suggest a smaller capacity for energy gain from food in vegetarians. Higher abundances of fibrolytic *Bacteroides* were found and might compensate for reductions in *Clostridium* cluster IV. This shift in these nutritionally important bacterial subgroups might account for the distinct grouping of omnivores and vegetarians in PCA of dominant bacterial fingerprints. In summary, it remains to be determined whether these shifts result in differential metabolite profiles that might in turn affect host metabolism and disease risks.

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References

1 Vinnari M, Montonen J, Harkanen T, Mannisto S: Identifying vegetarians and their food consumption according to self-identification and operationalized definition in Finland. Public Health Nutr 2009;12:481–488.

2 Mueller S, Saunier K, Hanisch C, Norin E, Alm L, Midtvedt T, Cresci A, Silvi S, Orpianesi C, Verdenelli MC, Clavel T, Koebnick C, Zunft HJ, Dore J, Blaut M: Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. Appl Environ Microbiol 2006;72:1027–1033.

3 Zwielehner J, Handschur M, Michaelsen A, Irez S, Demel M, Denner EB, Haslberger AG: DGGE and real-time PCR analysis of lactic acid bacteria in bacterial communities of the phyllosphere of lettuce. Mol Nutr Food Res 2008;52:614–623.

4 Scanlan PD, Shanahan F, Clune Y, Collins JK, O'Sullivan GC, O'Riordan M, Holmes E, Wang Y, Marchesi JR: Culture-independent analysis of the gut microbiota in colorectal cancer and polyposis. Environ Microbiol 2008;10:789–798.

5 Packey CD, Sartor RB: Interplay of commensal and pathogenic bacteria, genetic mutations, and immunoregulatory defects in the pathogenesis of inflammatory bowel diseases. J Intern Med 2008;263:597–606.

6 Li M, Wang B, Zhang M, Rantalainen M, Wang S, Zhou H, Zhang Y, Shen J, Pang X, Zhang M, Wei H, Chen Y, Lu H, Zuo J, Su M, Qiu Y, Jia W, Xiao C, Smith LM, Yang S, Holmes E, Tang H, Zhao G, Nicholson JK, Li L, Zhao L: Symbiotic gut microbes modulate human metabolic phenotypes. Proc Natl Acad Sci USA 2008;105:2117–2122.

7 Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmee E, Cousin B, Sulpice T, Chamontin B, Ferrieres J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, Burcelin R: Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007;56:1761–1772.

8 Suau A, Rochet V, Sghir A, Gramet G, Brewaeys S, Sutren M, Rigottier-Gois L, Dore J: *Fusobacterium prausnitzii* and related species represent a dominant group within the human fecal flora. Syst Appl Microbiol 2001;24:139–145.

9 Dethlefsen L, Eckburg PB, Bik EM, Relman DA: Assembly of the human intestinal microbiota. Trends Ecol Evol 2006;21:517–523.

10 Pryde SE, Duncan SH, Hold GL, Stewart CS, Flint HJ: The microbiology of butyrate formation in the human colon. FEMS Microbiol Lett 2002;217:133–139.

11 Louis P, Scott KP, Duncan SH, Flint HJ: Understanding the effects of diet on bacterial metabolism in the large intestine. J Appl Microbiol 2007;102:1197–1208.

12 Csordas A: Butyrate, aspirin and colorectal cancer. Eur J Cancer Prev 1996;5:221–231.

13 Thaler R, Karlic H, Rust P, Haslberger AG: Epigenetic regulation of human buccal mucosa mitochondrial superoxide dismutase gene expression by diet. Br J Nutr 2009;101:743–749.

14 Karlic H, Schuster D, Varga F, Klindert G, Lapin A, Haslberger A, Handschur M: Vegetarian diet affects genes of oxidative metabolism and collagen synthesis. Ann Nutr Metab 2008;53:29–32.

15 Guigoz Y, Dore J, Schiffrin EJ: The inflammatory status of old age can be nurtured from the intestinal environment. Curr Opin Clin Nutr Metab Care 2008;11:13–20.

16 Kirjavainen PV, Apostolou E, Arvola T, Salminen SJ, Gibson GR, Isolauri E: Characterizing the composition of intestinal microflora as a prospective treatment target in infant allergic disease. FEMS Immunol Med Microbiol 2001;32:1–7.

17 Muyzer G, de Waal EC, Uitterlinden AG: Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 1993;59:695–700.

18 Hayashi H, Sakamoto M, Benno Y: Fecal microbial diversity in a strict vegetarian as determined by molecular analysis and cultivation. Microbiol Immunol 2002;46:819–831.

19 Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI: The human microbiome project. Nature 2007;449:804–810.

20 Penders J, Vink C, Driessen C, London N, Thijs C, Stobberingh EE: Quantification of *Bifidobacterium* spp., *Escherichia coli* and *Clostridium difficile* in faecal samples of breast-fed and formula-fed infants by real-time PCR. FEMS Microbiol Lett 2005;243:141–147.

21 Layton A, McKay L, Williams D, Garrett V, Gentry R, Sayler G: Development of *Bacteroides* 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl Environ Microbiol 2006;72:4214–4224.

▶22 Yu Y, Lee C, Kim J, Hwang S: Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol Bioeng 2005;89:670–679.

▶23 Matsuki T, Watanabe K, Fujimoto J, Takada T, Tanaka R: Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. Appl Environ Microbiol 2004;70:7220–7228.

▶24 Edwards U, Rogall T, Blocker H, Emde M, Bottger EC: Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res 1989;17:7843–7853.

▶25 Heuer H, Hartung K, Wieland G, Kramer I, Smalla K: Polynucleotide probes that target a hypervariable region of 16S rRNA genes to identify bacterial isolates corresponding to bands of community fingerprints. Appl Environ Microbiol 1999;65:1045–1049.

▶26 Neefs JM, Van de Peer Y, De Rijk P, Goris A, De Wachter R: Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res 1991;19(suppl):1987–2015.

▶27 Bernhard AE, Field KG: A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA. Appl Environ Microbiol 2000;66:4571–4574.

▶28 Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, Oyaizu H, Tanaka R: Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl Environ Microbiol 2002;68:5445–5451.

▶29 Shen J, Zhang B, Wei G, Pang X, Wei H, Li M, Zhang Y, Jia W, Zhao L: Molecular profiling of the *Clostridium leptum* subgroup in human fecal microflora by PCR-denaturing gradient gel electrophoresis and clone library analysis. Appl Environ Microbiol 2006;72:5232–5238.

▶30 Muyzer G, Smalla K: Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 1998;73:127–141.

▶31 Stephen AM, Wiggins HS, Cummings JH: Effect of changing transit time on colonic microbial metabolism in man. Gut 1987;28:601–609.

▶32 Silk DB, Walters ER, Duncan HD, Green CJ: The effect of a polymeric enteral formula supplemented with a mixture of six fibres on normal human bowel function and colonic motility. Clin Nutr 2001;20:49–58.

▶33 MacFarlane GT, Gibson GR: Co-utilization of polymerized carbon sources by *Bacteroides ovatus* grown in a two-stage continuous culture system. Appl Environ Microbiol 1991;57:1–6.

▶34 Salyers AA: Bacteroides of the human lower intestinal tract. Annu Rev Microbiol 1984;38:293–313.

▶35 Narushima S, Ito K, Kuruma K, Uchida K: Composition of cecal bile acids in ex-germfree mice inoculated with human intestinal bacteria. Lipids 2000;35:639–644.

▶36 Mueller S, Saunier K, Hanisch C, Norin E, Alm L, Midtvedt T, Cresci A, Silvi S, Orpianesi C, Verdenelli MC, Clavel T, Koebnick C, Zunft HJ, Dore J, Blaut M: Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. Appl Environ Microbiol 2006;72:1027–1033.

13.2. Published research article – Combined PCR-DGGE fingerprinting and quantitiative-PCR indicates shifts in fecal population sizes and diversity of Bacteroides, bifidobacteria and *Clostridium* cluster IV

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Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of *Bacteroides*, bifidobacteria and *Clostridium* cluster IV in institutionalized elderly

Jutta Zwielehner [a], Kathrin Liszt [a], Michael Handschur [a], Cornelia Lassl [a], Alexander Lapin [b], Alexander G. Haslberger [a,*]

[a] *Department of Nutritional Sciences, University of Vienna, Austria*
[b] *Sozialmedizinisches Zentrum Sophienspital, 1070 Wein, Apollogasse 19, Austria*

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ABSTRACT

Aims: This study aimed at determining ageing-related shifts in diversity and composition of key members of the fecal microbiota by comparing institutionalized elderly (n = 17, 78–94 years) and young volunteers (n = 17, 18–31 years).
Methods and results: A combination of molecular methods was used to characterize the diversity and relative abundance of total gastro-intestinal flora, along with relevant subsets within the *genera Bacteroides*, bifidobacteria and *Clostridium* cluster IV. The institutionalized elderly harbored significantly higher numbers of *Bacteroides* cells than control (28.5 ± 8.6%; 21.4 ± 7.7%; p = 0.016) but contained less bifidobacteria (1.3 ± 0.9, 2.7 ± 3.2%, p = 0.026) and *Clostridium* cluster IV (26.9 ± 11.7%, 36.36 ± 11.26%, p = 0.036). The elderly also displayed less total *Bacteria* diversity and less diversity with the *Clostridium* cluster IV (p < 0.016) and *Bacteroides*.
Conclusion: Despite high individual variations, our analyses indicate the composition of microbiota in the elderly comprises a less diverse subset of young healthy microbiota.
Significance and impact of the study: A better understanding of the individual composition of the human microbiota and the effects of ageing might result in the development of specifically targeted supplementation for elderly citizens in order to support healthy ageing.

1. Introduction

It is estimated that between 60% and 80% of the microbial diversity resident within the human gastro-intestinal tract (GI) has yet to be cultivated (Suau et al., 1999). However, the use of culture-independent molecular methods has enabled researchers to identify important characteristics of this community. We now know that the composition of GI microbiota is highly distinct between individuals (Dethlefsen et al., 2006). Despite this variability, some general features are apparent. There appear to be 'core species' present in a majority of humans which are quite resilient to external influences (Dore, 2007). In addition to this group, there are also 'passengers' or transients, sometimes in great numbers, sometimes below detection limit (Favier et al., 2003). However, the majority of sequences seem to be unique to some individual and not one single phylotype may be present in all humans (Turnbaugh et al., 2009). A number of factors have been identified which influence community composition. The type of bacteria introduced to the environment is determined by those associated with food sources (Duncan et al., 2007; Pryde et al., 2002). The nature and composition of non-digestible carbohydrates in a diet can further stimulate different types of bacteria (Kolida and Gibson, 2007), whilst hereditary dispositions and personal gut environmental factors may account for unique personal characteristics (Eckburg and Relman, 2007).

Correlations between the GI microbiota and diseases such as allergenicity (Shreiner et al., 2008), inflammatory bowel diseases (Sokol et al., 2006) and individual dispositions such as obesity (Zhang et al., 2009) have been discussed.

The colon harbors butyrate-producing species of several genera such as *Clostridium, Eubacterium* and *Fusobacterium* (Pryde et al., 2002). Among strains that produce high levels of butyrate *in vitro* are those related to *Faecalibacterium prausnitzii*, an ubiquitous member of *Clostridium* cluster IV (Pryde et al., 2002). *Bacteroidetes* species have been reported to show high variations between individuals, although *Bacteroides thetaiotaomicron* is found in all human beings

Abbreviations: APS, ammonium-persulfate; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; GIT, gastro-intestinal tract.

* Corresponding author. Address: Univ.-Doz. Dr. Alexander Haslberger, Department of Nutritional Sciences, Althanstrasse 14, UZAII;2D541, 1090 Vienna, Austria. Tel.: +431 427754996; fax : +431 42779549.
E-mail address: alexander.haslberger@univie.ac.at (A.G. Haslberger).

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(Layton et al., 2006). Both *Bacteroidetes* and *Clostridium* cluster IV are known to be involved in beneficial functions, including nutrient absorption, production of short chain fatty acids (SCFAs) and epithelial cell maturation and maintenance (Woodmansey, 2007).

Another important subgroup of the human GI microbiota is the bifidobacteria. Stimulation of these bacteria has previously been shown after prebiotic intervention with inulin and fructo-oligosaccharides (Kolida and Gibson, 2007). Furthermore, they have been shown to be involved in prevention of atopic disease (Ouwehand, 2007), obesity and insulin resistance via enhanced barrier function of the gut epithelium (Cani et al., 2007).

With ageing, a decrease in beneficial organisms such as *Lactobacilli* and bifidobacteria, amongst other anaerobes, and an increase in the number of facultative anaerobes (Guigoz et al., 2008) have been reported. Population cross-sectional studies with relevant numbers of healthy elderly also show age-related changes in GI microbiota. These include a consistent global increase in nonpathogenic Gram-negative bacteria (mainly *Enterobacteria*), as well as country-specific changes in bifidobacteria (Guigoz et al., 2008). This, along with a general reduction in species diversity within most bacterial groups, changes to diet and altered digestive physiologies such as intestinal transit time, may result in increased putrefaction in the colon and a greater susceptibility to disease. The aged gut is characterized by increased proteolytic activity, decreased amylolytic activity and reduced levels of SCFA (Woodmansey, 2007). Ageing is associated with reduced levels of prostaglandins such as PGE_2 and $PGF_2\alpha$ as shown in specimens of stomach and duodenum biopsies (Tiihonen et al., 2008). Subclinical intestinal inflammation in elderly populations has been detected and is believed to contribute to impaired immune functions, the underlying cause of mortality beyond 75–80 years of age (Guigoz et al., 2008).

Analyses of individual dispositions associated with changes of the microbiota should consider quantitative and qualitative aspects of gut community structure. Analysis of stool samples can target changes in colonic microbiota, since feces are representative of inter-individual differences (Turnbaugh et al., 2007).

The aim of this work was to investigate shifts in GI microbiota associated with ageing, by comparing institutionalized elderly with young healthy volunteers. To analyze changes in total bacterial community composition, along with specific compositional changes within the *Bacteroidetes*, bifidobacteria and *Clostridia* cluster IV, we used the polymerase chain reaction (PCR) based community fingerprinting method Denaturing Gradient Gel Electrophoresis. The resolution of this method allows for the characterization of the dominant members of a targeted microbial community. Further, we used quantitative-PCR (q-PCR) to determine the relative load of *Bacteriodetes*, bifidobacteria and *Clostridia* cluster IV groups within our samples. Thus, use of these methods in combination allowed the characterization of both diversity and relative abundance of our targeted organisms.

2. Material and methods

2.1. Probands

Seventeen institutionalized elderly aged 86 ± 8 years, BMI 21.75 ± 5.08, from a geriatric department in Vienna and 17 students from Vienna joined the study. Probands were interviewed following a questionnaire assessing: age; gender; body length and weight; individual health status, including chronic or acute diseases and blood lipid levels; and life-style aspects, such as physical activity and dietary habits. Five percent of geriatric patients suffered from manifest diabetes mellitus type 2. Ten patients were bed-ridden and seven mobile. Causes for loss of mobility were Parkinson's disease, dementia and osteoporosis. Nursing staff reported the application of NSAIDs (non-steroidal anti-inflammatory drugs) on demand. Seventeen young healthy volunteers were aged 24 ± 2.5 years, BMI 22.68 ± 3.41 and their dietary habits were typical for Central Europe.

Study populations were gender balanced, with 55% females in the group of elderly and 50% in the young group. Only non-pregnant probands with no diagnosed gastro-intestinal disease and no antibiotic or chemotherapeutic treatment three months prior to sampling were included in the study. All probands agreed to participate in the study and gave their informed consent.

2.2. Sampling and DNA extraction from stools and type strains

From each proband, three stool samples were taken within the course of a week and immediately stored at −70 °C. Portions of the three samples from each patient were pooled, a 200 mg aliquot was treated twice for 45 s in a bead-beater (Mini-Beadbeater-8) and DNA extracted with the QIAamp® DNA Stool Mini Kit (QIAGEN) following the manufacturer's protocol and then immediately stored at −20 °C.

Type strains known to be associated with GI microbiota were grown, and DNA extracted, for use as part of the markers in DGGE analysis (see below). Type strains *L. casei* DSM 20011^T, *L. delbrueckii* subsp. *lactis* DSM 20072^T, *Bacteroides fragilis* DSM 2151^T, *B. thetaiotaomicron* DSM 2079^T, *Bifidobacterium longum* DSM20219^T, *B. longum* DSM 20211, *Bifidobacterium pseudolongum* DSM 20099 and *Bifidobacterium thermophilus* DSM 20210 were anaerobically cultivated on blood agar, *Escherichia coli* IMBH 252/07 and clones were aerobically cultivated on LB-agar (liquid broth medium). The biomass was resuspended in sterile phosphate buffered saline (1× PBS from 10× PBS Roti®stock, ROTH). Tenfold dilutions from these suspensions in sterile 1x PBS were plated in duplicate on blood agar or LB-agar, colony forming units (CFU/ml) were counted and DNA extracted from serial dilutions. DNA was extracted from clones using the Wizard® Plus.SV Minpreps DNA Purification System (Promega). The DNA of all Gram-negative bacteria was extracted with the DNA Mini Kit (QIAGEN), for Gram- positive bacteria, the FastDNA Spin Kit for Soil (MP-Biomedicals) was used following the instructions of the manufacturer.

2.3. Polymerase chain reaction (PCR)

PCR was used to amplify 16S ribosomal RNA gene sequences from type strains and *Bacteria* in stool samples for use in DGGE analysis and manufacture of clone libraries (see below). All reactions were carried out using a ready-to-use mastermix (Promega) with 1.5 mM $MgCl_2$. Bovine serum albumin (10 mg/ml, Fermentas) was added to a final concentration of 400 μg/ml, primer concentration in the reaction volume was 0.5 μM. Amplifications were carried out in a Robocycler (Stratagene).

2.4. Clone libraries

Clone libraries were constructed from stool samples to identify dominant members of the *Clostridium* cluster IV and the *Bacteroidetes*. Selected clones were then used, along with the cultured type strains, to generate appropriate reference markers for DGGE analysis. Amplifications were carried out using primer pair 27f Edwards et al., 1989) and sg-Clep-R (Matsuki et al., 2004), which are specific for members of the *Clostridium* cluster IV, and 32f and 708r (Bernhard and Field, 2000) which are specific for members of the *Bacteroides*. Amplified products were cloned into a p-GEM Easy Vector (Promega) following the instructions of the manufacturer. Clone libraries were screened as previously described (Schabereiter-Gurtner et al., 2001). Clone inserts were sequenced by 'DNA confidence' (Vienna). Nucleotide sequences were corrected for vector and primer sequences in CodonCode

Table 1
Primers and probes used for quantification of fecal bacteria using TaqMan assays targeting 16S rRNA coding regions.

Target organism	Primer or probe	Sequence (5′–3′)	Fragment size (bp)	Conc. (nM)	Reference
Bifidobacteria	Fwd primer	GCG TGC TTA ACA CAT GCA AGT C	125	300	
	Rev primer	CAC CCG TTT CCA GGA GCT ATT		300	
	Probe	(FAM)- TCA CGC ATT ACT CAC CCG TTC GCC -(BHQ-1)		150	Penders et al. (2005)
Bacteroides	AllBac296f	GAG AGG AAG GTC CCC CAC	106	300	
	AllBac412r	CGC TAC TTG GCT GGT TCA G		300	
	AllBac375Bhqr	(FAM)-CCA TTG ACC AAT ATT CCT CAC TGC TGC CT-(BHQ-1)		100	Layton et al. (2006)
Bacteria (general)	BAC-338-F	ACT CCT ACG GGA GGC AG	468	1000	
	BAC-805-R	GAC TAC CAG GGT ATC TAA TCC		1000	
	BAC-516-P	(FAM)-TGC CAG CAG CCG CGG TAA TAC-(BHQ-1)		200	Yu et al. (2005)
Clostridium cluster IV	sg-Clep-F	GCA CAA GCA GTG GAG T	239	400	
	sg-Clep-R	CTT CCT CCG TTT TGT CAA		400	Matsuki et al. (2004)
	Clep-P[a]	(FAM)-AGG GTT GCG CTC GTT-(BHQ-1)		200	This study

[a] Position of target site (numbering corresponding to *E. coli* 16S rRNA gene) 1082–1107.

Aligner (www.codoncode.com) and taxonomically identified by comparison to previously published sequences using the online tools of the ribosomal database project (http://rdp.cme.msu.edu/).

2.5. DGGE

PCR amplifications of 16S rRNA gene fragments from total DNA extracted from stool samples were carried out using the specific primer sets outlined in Table 2.

Separate DGGE gels were run to analyze samples for patterns in (a) total *Bacteria*, (b) bifidobacteria, (c) *Clostridium* cluster IV and (d) *Bacteroides*. DGGE gels were prepared as described previously (Muyzer and Smalla, 1998) with a linear gradient of 25–65% for *Bacteria* (general), 30–65% for bifidobacteria, 20–50% for *Bacteroides* and 30–50% for *Clostridium* cluster IV, using a gradient mixer (Hoefer SG 30) and a peristaltic pump. We generated reference markers appropriate for each set of DGGE analyses (i.e., a different reference marker was used for each of a, b, c and d above) to enable meaningful comparisons across multiple gels and to provide putative identification of some bands. These reference markers contained fragments of 16S rRNA genes from cultured bacteria and clones generated from fecal material (as described above). Each marker was loaded in triplicate on each gel to allow gel-to-gel comparison. The reference marker for DGGE fingerprinting of general *Bacteria* consisted of *E. coli* IMBH 252/07, *Enterococcus faecium* DSM 20477^T, *B. thetaiotaomicron* DSM 2079^T, *B. longum* DSM 20219^T, *Clostridium perfringens* (laboratory isolate), clone BT11 (98.8% similarity with *Bacteroides uniformis* JCM 5828^T), clone BT17 (96.0% similarity with *Bacteroides vulgatus*^{T} and bacterium LY88 previously identified in human feces (Eckburg et al., 2005)) and clone CL16 (98.4% similarity with an uncultured bacterium from human feces (Turnbaugh et al., 2006) and 96.7% similarity with *F. prausnitzii*^{T}). The reference marker for bifidobacteria specific DGGE analysis consisted of *E. faecium* DSM 20477^T, *E. coli* IMBH 252/07, *B. longum* DSM 20219^T, *B. longum* DSM 20211, *B. thermophilus* DSM 20210^T and *B. pseudolongum* DSM 20099^T. The reference marker for *Bacteroides* specific DGGE analysis was composed of *B. thetaiotaomicron* DSM 2079^T, fecal clones Bt 17 and Bt11 and *E. faecium* DSM 20477^T. The reference marker for *Clostridium* cluster IV DGGE analysis was constructed from 7 clones all representing previously uncultured species from cluster IV: Similarities with type strains from this cluster were 81.8%, 96.6%, 96.7% and 97.8% similarity with *F. prausnitzii*^{T}, 92.1% and 92.9% similarity with *Eubacterium desmolans*^{T} and 95.9% similarity with *Subdoligranulum variabile*^{T}.

2.6. TaqMan q-PCR

The TaqMan-assay was carried out in a Rotorgene 3000 (Corbett Life Science) in duplicate in a volume of 10 µl containing 5 µl TaqMan SensiMix DNA Kit (Quantace), 1 µl of each primer and probe (final concentrations Table 1) and 2 µl of the 100-fold dilution of the template. All probes were labeled with 6-FAM at the 5′ end and carried a BHQ-1 quencher at the 3′ end. TaqMan Probe (Clep-P) for *Clostridium* cluster IV was designed with CLC DNA Workbench (www.clcbio.com). Analysis with ProbeMatch (rdp.9.58) indicated that Clep-P binds to all members of *Clostridium* cluster IV. The PCR program for bifidobacteria, *Bacteroides* and universal bacteria consisted of denaturing at 95 °C for 3 min and 45 cycles of 95°/60 °C for 15/45 s. The amplification program for clostridium cluster IV was: denaturation at 95° for 5 min and 45 cycles at 95°/55 °C for 30/45 s.

DNA of *B. thetaiotaomicron*^{T} and *B. longum*^{T}, clone CL16 and one fecal sample were used to construct standard curves for comparison of PCR reaction efficiencies among different experiments and enumeration of all bacterial groups. Reaction efficiency (E) was

Table 2
Primers applied for PCR-DGGE fingerprinting of 16S rRNA coding regions.

Target organism	Primer pairs	Sequence (5′–3′)	Ann. temp (°C)	Reference
Bacteria	27f	GTGCTGCAGAGAGTTTGATCCTGGCTCAG	57	Edwards et al. (1989)
	985r	GTAAGGTTCTTCGCGTT	57	Heuer et al. (1999)
	341f-GC	CCT ACG GGA GGC AGC AG	55	Muyzer et al. (1993)
	518r	ATT ACC GCG GCT GCT GG	55	Neefs et al. (1991)
Bacteroides	32f	AACGCTAGCTACAGGCTT	56	Bernhard and Field (2000)
	708r	CAATCGGAGTTCTTCGTG	56	Bernhard and Field (2000)
Bifidobacteria	g-BifidF	CTCCTGGAAACGGGTGG	58	Matsuki et al. (2002)
	g-BifidR	GGTGTTCTTCCCGATATCTACA	58	Matsuki et al. (2002)
Clostridia cluster IV	sg-Clep-F-GC sg-Clep-R	see Table 1	55	

estimated using the slope of the standard curve and the formula $E = 10^{(-1/\text{slope})} - 1$ as described elsewhere (Penders et al., 2005). Quantification was done using standard curves obtained from known concentrations of organisms containing the respective amplicons for each set of primers. The percentage of bacterial group rRNA gene copies in relation to total rRNA gene copies (relative abundance) was calculated for each individual, and the mean was determined for each subject group. Relative quantification (percentage of bacteria) was performed using Rotor-Gene 3000 calculation software (Corbett operator manual) and Excel. Cross reactivity with non-target strains was tested using the Probe-Match tool at the RDP website and using the strains mentioned above.

2.7. Statistical analysis

Food frequency data were analyzed based on χ-square approximation as implemented in SPSS. *P* values <0.05 were considered significant. Band comparison tables were analyzed with principal component analysis (PCA) using the default settings in 'R-software environment for statistical computing' (www.r-project.org) until 100% variance was explained. Transformed data were plot in a bi-plot as a function of the first two principal components. Clustering was applied to DGGE fingerprinting data as implemented in the GelComparII environment (www.applied-maths.com). Clustering was performed based on Dice coefficient as well as based on Pearson correlation. Those methods are supplementary to each other, clustering after Dice takes band positions into account, whereas Pearson correlation based clustering analyzes the densitometric curves of each fingerprint. UPGMA dendrograms were generated and Jackknife analysis was performed using average similarities and 100 resamplings. Jackknife analysis is a leave-one-out method that tests the reliability of the clustering similar to bootstrap analysis. Shannon and Simpson's diversity index were calculated on binary band information (presence–absence) with the default settings implemented in the 'vegan' package in '*R*'. Shannon index is defined as $H = -\sum p_i \ln p_i$, where p_i is the proportional abundance of species *i*. For Simpson's index *D*, this is $1 - \sum((p_i)\text{^}2)$, where p_i is the relative frequency of the *i*-th species. Prior to application of Student's *t*-test for diversity indices and qPCR data, equality of variances of the three datasets was tested using the *F*-test as implemented in Microsoft Excel.

3. Results

3.1. Dietary aspects

Analysis of the participant's dietary habits indicated similar consumption patterns of fruits, vegetables and milk products in both groups. Young volunteers stated significantly less frequent (χ^2 Test; $p < 0.04$) consumption of meat than elderly probands and regular consumption of whole grain products several times a week. The institutionalized elderly of this study did not consume any whole grain products at all but received supplements with soluble fiber (Benfiber®, Novartis).

3.2. TaqMan-quantification

TaqMan assays were set up quantifying bacterial sub-populations as percentage of the total bacterial DNA. We detected no cross-reactivity of group-specific primers and probes with non-target strains. Test-retest variations were between 2.7% and 5.2%, values after relative quantification varied by less than 4%. The sensitivity was corresponding to a 100,000-fold dilution of DNA from feces, that is, 20 copies of 16S rRNA gene per reaction.

The elderly harbored only 69 ± 21.6% of the total bacterial load in their feces compared to control (Fig. 1). *Bacteroides* were found to represent a larger percentage than bifidobacteria in all samples (Fig. 1) and the institutionalized elderly harbored significantly more *Bacteroides* than young volunteers ($p = 0.016$). Although relative levels of bifidobacteria were highly variable among samples, the differences between young and elderly probands were statistically significant ($p = 0.026$). Furthermore, elderly citizens had significantly less members of *Clostridium* cluster IV (Fig. 1) in their fecal microbiota than young volunteers ($p = 0.036$).

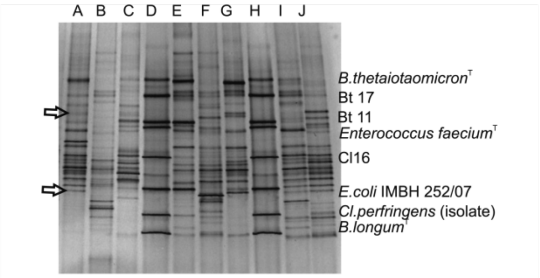

Fig. 2. PCR-DGGE bandpattern of 16S rRNA coding regions of dominant bacteria amplified with primer pair 341GC-518. Bands that were observed more frequently in young than in elderly are indicated with arrows. Organisms and sequences listed were used for the construction of the reference lanes. A, G, I, J, young; B, C, E, F, elderly; D, H, reference lanes.

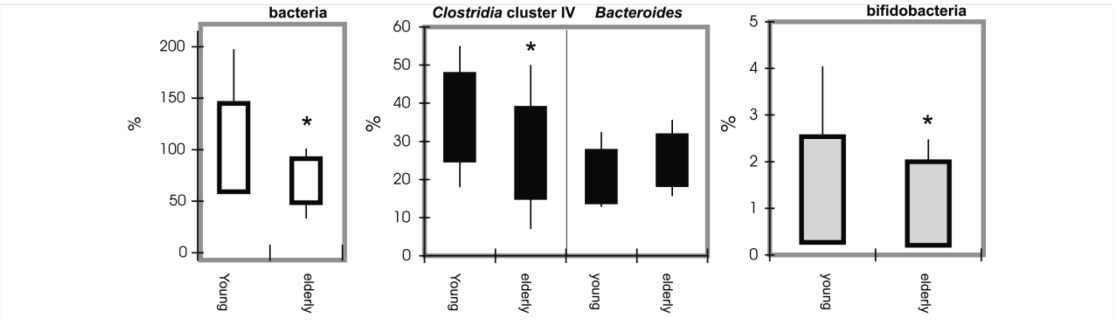

Fig. 1. Proportions of *Bacteroides*, bifidobacteria and *Clostridium* cluster IV of the total bacterial load in stool samples of institutionalized elderly and young healthy volunteers. Total amount of bacteria is depicted relative to the mean counts for healthy young young.

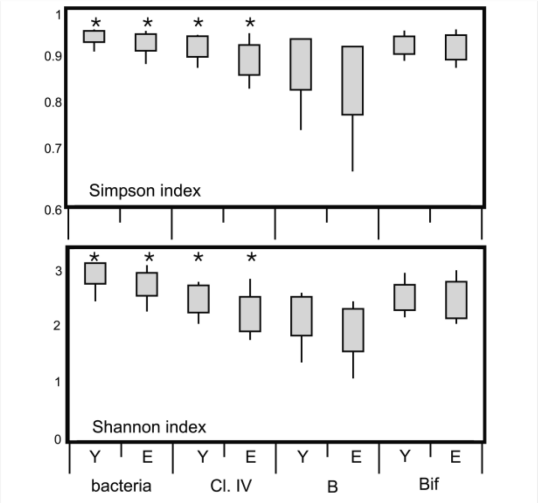

Fig. 3. Diversity indices derived from DGGE fingerprinting of 16S rRNA coding regions. Y, young; E, elderly; Cl.IV, *Clostridium* cluster IV; B, *Bacteroides*, Bif, bifidobacteria; *, $p < 0.05$.

3.3. DGGE bandpattern analysis

DGGE fingerprinting with primer pair 341GC-518, which amplified the total microbial community, showed high inter-individual variations (Fig. 2). The mean numbers of bands per individual were 16.6 ± 3 bands for institutionalized elderly and 20 ± 3 bands in control. Two bands, highlighted in Fig. 2, occurred significantly less frequently in elderly probands. Shannon and Simpson indices of diversity were significantly lower for elderly than control (Fig. 3). The highly diverse dataset was subjected to principal component analysis (PCA). Extraction of underlying components within the dataset indicated grouping of samples according to their variance (Fig. 4) along the first two principal components. Cluster analysis could separate the fingerprints of young and elderly (Fig. 5). Jackknife analysis suggested that the dominant bacteria of the elderly microbiota are a subpopulation of the microbiota of young individuals: Predictability of groupings was 100% for young and only 13.33% for elderly.

3.3.1. Bifidobacteria

An average of 13 bands were observed in individual DGGE bandpatterns obtained with the primer pair specific for bifidobacteria. Two bands with the same melting behaviour as the type strains *B. longum* DSM 20219^T and *B. pseudolongum* DSM 20099^T in our marker were abundant in the majority of probands. PCA was performed and the first two PC's explained 23.64% of variance (Fig. 4). Although cluster analysis showed high similarity of young and elderly bandpatterns, jackknife testing demonstrated greater similarity of bandpattern for control than for elderly (expressed in predictability of groupings: 91.67% for control and 35.72% for elderly). PCA supported these results and could not separate young and elderly according to variances in the dominant bifidobacteria of their microbiota.

3.3.2. Bacteroides

DGGE fingerprints obtained with the *Bacteroides* specific primers contained an average of 7.6 ± 2.5 bands for the elderly, whilst young individuals averaged 9.5 ± 3 bands. Diversity indices (Fig. 3) showed a tendency to be lower for elderly than for control. PCA results suggest a tendency for less *Bacteroides* diversity with ageing (Fig. 4). Elderly subjects grouped along the first principal component. Clustering of Pearson correlations could separate young and elderly individuals according to DGGE fingerprinting. Jackknife analysis using average similarities could predict groupings of young bandpattern with 76.92% reliability and 75% for elderly.

3.3.3. Clostridium cluster IV

DGGE fingerprints obtained with the *Clostridium* cluster IV specific primers yielded an average of 10 ± 3.5 bands per elderly individual and 13 ± 3 bands per young individual. Diversity indices (Fig. 2) were significantly ($p = 0.02$) lower for aged citizens than for the young. One band that occurred more frequently in the young than in the elderly had the same melting characteristics as a band in our marker. This band was generated from a clone from our library related to the genus *Faecalibacterium*. This clone had the highest similarity (99.2%) to an uncultured bacterium EF403698 and also displayed 96.6% similarity to *F. prausnitzii*^{T}. Three more bands occurred more frequently in the young than in elderly. These bands were identified from our clone libraries as relating to the genera *Ruminococcus* (clone had 95.9% similarity to *Ruminococcus bromii*^{T}, 99% similarity to uncultured AJ408987 from human colon) and *Subdoligranulum* (clones had 94.7%/97.5% similarity to *S. variabile*^{T}, and 97.9%/98% similarity to uncultured bacterium DQ793301).

PCA (Fig. 4) indicated separation of young and elderly according to their *Clostridium* cluster IV DGGE fingerprints along PC1.

Clustering and Jackknife analysis did not result in distinct clustering of elderly and young individuals. *Clostridium* cluster IV representatives in the elderly microbiota are most likely to be a subset of the species present in young individuals: Only in 35.71% of

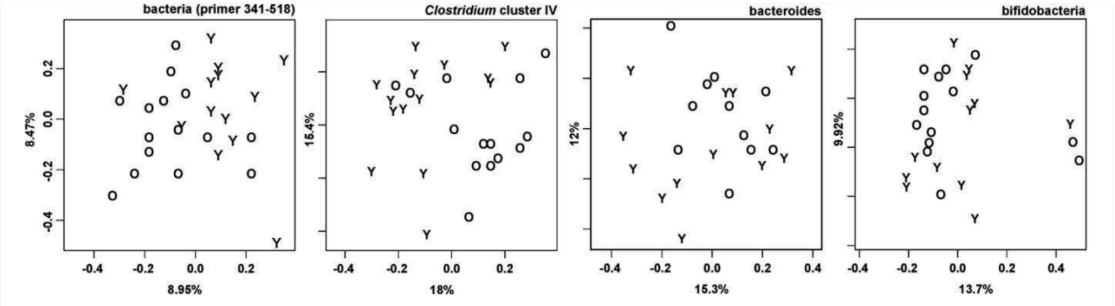

Fig. 4. PCA of DGGE fingerprints of 16S rRNA coding regions of dominant bacteria in fecal samples. Y, young; O, elderly.

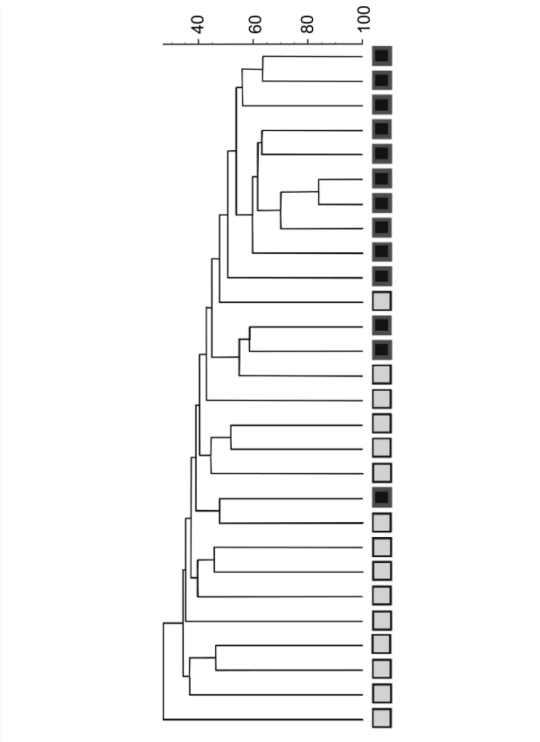

Fig. 5. UPMGA dendrogram showing clustering (Dice) based on the similarities of DGGE fingerprints of dominant bacteria obtained with primer pair 341GC-518. ☐ elderly, ■ young.

resamplings elderly bandpattern grouped with each other, whereas Jackknife value for young individuals was 100%.

4. Discussion

Metagenomic analysis of the human GI microbiota is presently the subject of large research consortia and has already substantiated the concepts of a 'core microbiome' and inter-individual variations (Turnbaugh et al., 2007). However, the collection of data on 'reference microbiota' is far from complete and thus no definition of a healthy microbiota is available yet. Important information comes from analyses addressing the abundance and diversity of specific bacterial populations with relevance to disease, diet or probiotic intervention. We used a combined molecular approach to compare patterns in several target GI microbial groups between the institutionalized elderly and young healthy volunteers. The community fingerprinting method PCR-DGGE was used to compare the diversity present in total *Bacteria* and also specifically within the *Clostridium* cluster IV, bifidobacteria and *Bacteroides*, whilst q-PCR was used to quantify the relative population abundance of these same bacterial groups in all samples.

Our results demonstrate some significant shifts in patterns in GI microbiota between our study groups. Faeces from the institutionalized elderly had less total Bacterial abundance and lower total Bacterial diversity than that from the young subjects, However, samples from the elderly displayed an increase in the relative abundance of *Bacteroides*, although this group tended to display less diversity than *Bacteroides* in the young. The relative abundance of the bifidobacteria and *Clostridium* cluster IV were significantly higher in the young, and the *Clostridium* cluster IV also displayed greater diversity in the young. Furthermore, cluster analysis revealed that for all microbial groups analyzed, the members of the GI microbiota in the elderly could be considered a subset of that present in the young.

The results of our bifidobacteria analysis are in agreement with previous studies which have identified a reduction and loss of diversity of bifidobacteria associated with ageing (Woodmansey et al., 2004; Hopkins et al., 2002) or hospitalization of aged citizens (Bartosch et al., 2004). Our study population of elderly was supplemented with soluble fiber. This prebiotic intervention alone was apparently not able to antagonize ageing-related changes in the bifidobacteria. In this respect Ouwehand et al. (2008) recently reported that supplementation with lactitol and a probiotic *Lactobacillus* led to an increase in bifidobacteria in the microbiota of elderly.

Bartosch et al. (2004) also reported a marked reduction in the abundance of *F. prausnitzii*, a member of the *Clostridium* cluster IV which decreased in our aged group. However several studies (Hopkins et al., 2002; Woodmansey et al., 2004; Bartosch et al., 2004) have reported a decrease in the relative abundance of the *Bacteroides*, whereas our aged study population displayed a relative increase in abundance of this group. Like us, all the previous studies reported a decrease in *Bacteroides* diversity. Increased levels of *Bacteroides* have been found in individuals with infectious colitis (Sokol et al., 2006). Discrepancies in changes of the *Bacteroides* abundance might be due to country-specific differences in this bacterial subgroup as indicated by Mueller et al. (2006). Mueller et al. (2006) also observed gender effects within *Bacteroides*, with levels being generally higher in males than in females. Our study populations were gender balanced with volunteers being 55% females in the group of elderly and 50% in the young. The results presented here do not support gender differences in *Bacteroides* abundance. Reductions in amylolytic activity observed in a healthy elderly population have been correlated with the occurrence and diversity of *Bacteroides* (Woodmansey, 2007).

Reduced numbers of *Bacteria* in the fecal content of elderly reflect the physiological alterations associated with ageing. These include prolonged colonic transit time and reduced dietary energy requirement and food uptake (Morley, 2007). Further reductions in the relative abundance of important sub-populations such as *Clostridium* cluster IV and bifidobacteria might result in reduced formation of SCFAs, altered epithelial cell maturation and maintenance, and altered barrier function of the gut epithelium in elderly probands. Those changes in the GI microbiota have previously been linked to impaired immune functions prevalent in individuals of advanced age and may result in a greater susceptibility to disease.

Improved analytical concepts for the characterization of the microbiota of consumers and patients might become important as a rationale for individualized probiotic intervention. Probiotic supplementation is a promising concept in restoring impaired functions or enhancing specific desirable functions of the microbiota. Encouraging effects of probiotic supplementation have been reported for aspects such as direction of host immunity, pathogen defense, maintenance of integrity of the gut epithelium, alleviation of lactose intolerance symptoms and immune effects, such as in atopic disease. For further insight into the relationships between phylogenetic information and metabolic activities, sequence information in addition to 16S rRNA based fingerprinting will be mandatory.

5. Conclusion

Studies comparing elderly and young volunteer microbiota with PCR-DGGE fingerprinting and q-PCR are still rare. We found that ageing is associated with less overall bacteria and significantly

decreased *Clostridium* cluster IV and bifidobacteria and an increase of *Bacteroides*. Diversity of dominant bacteria, *Bacteroides* and *Clostridia* cluster IV were reduced. Those changes in the GI microbiota are suggested to be cause and effect of impaired immune functions in individuals of advanced age and may result in a greater susceptibility to disease.

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References

Bartosch, S., Fite, A., Macfarlane, G.T., McMurdo, M.E., 2004. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl. Environ. Microbiol. 70, 3575–3581.

Bernhard, A.E., Field, K.G., 2000. A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides–Prevotella* genes encoding 16S rRNA. Appl. Environ. Microbiol. 66, 4571–4574.

Favier, C.F., de Vos, W.M., Akkermans, A.D., 2003. Development of bacterial and bifidobacterial communities in feces of newborn babies. Anaerobe 9 (5), 219–229.

Dethlefsen, L., Eckburg, P.B., Bik, E.M., Relman, D.A., 2006. Assembly of the human intestinal microbiota. Trends Ecol. Evol. 21, 517–523.

Dore, J. 2007 Gut microbiota in health and disease. In: Report of the 2nd International Workshop in Amsterdam, April 26–28.

Duncan, S.H., Louis, P., Flint, H.J., 2007. Cultivable bacterial diversity from the human colon. Lett. Appl. Microbiol. 44, 343–350.

Eckburg, P.B., Relman, D.A., 2007. The role of microbes in Crohn's disease. Clin. Infect. Dis. 44, 256–262.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., et al., 2005. Diversity of the human intestinal microbial flora. Science 308, 1635–1638.

Edwards, U., Rogall, T., Blocker, H., Emde, M., Bottger, E.C., 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. 17, 7843–7853.

Cani, P.D., Neyrinck, A.M., Fava, F., Knauf, C., Burcelin, R.G., Tuohy, K.M., Gibson, G.R., Delzenne, N.M., 2007. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia 50 (11), 2374–2383.

Guigoz, Y., Dore, J., Schiffrin, E.J., 2008. The inflammatory status of old age can be nurtured from the intestinal environment. Curr. Opin. Clin. Nutr. Metab. Care 11, 13–20.

Heuer, H., Hartung, K., Wieland, G., Kramer, I., Smalla, K., 1999. Polynucleotide probes that target a hypervariable region of 16S rRNA genes to identify bacterial isolates corresponding to bands of community fingerprints. Appl. Environ. Microbiol. 65, 1045–1049.

Hopkins, M.J., Sharp, R., Macfarlane, G.T., 2002. Variation in human intestinal microbiota with age. Dig. Liver Dis. 34 (Suppl. 2), S12–S18.

Kolida, S., Gibson, G.R., 2007. Prebiotic capacity of inulin-type fructans. J. Nutr. 137, 2503S–2506S.

Layton, A., McKay, L., Williams, D., Garrett, V., Gentry, R., Sayler, G., 2006. Development of Bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl. Environ. Microbiol. 72, 4214–4224.

Matsuki, T., Watanabe, K., Fujimoto, J., Takada, T., Tanaka, R., 2004. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. Appl. Environ. Microbiol. 70, 7220–7228.

Matsuki, T., Watanabe, K., Fujimoto, J., Miyamoto, Y., Takada, T., Matsumoto, K., et al., 2002. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl. Environ. Microbiol. 68, 5445–5451.

Morley, J.E., 2007. The aging gut: physiology. Clin. Geriat. Med. 23, 757–767. v–vi.

Mueller, S., Saunier, K., Hanisch, C., Norin, E., Alm, L., Midtvedt, T., et al., 2006. Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. Appl. Environ. Microbiol. 72, 1027–1033.

Muyzer, G., Smalla, K., 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 73, 127–141.

Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

Neefs, J.M., Van de Peer, Y., De Rijk, P., Goris, A., De Wachter, R., 1991. Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res. 19, 1987–2015.

Ouwehand, A.C., 2007. Antiallergic effects of probiotics. J. Nutr. 137 (3 Suppl. 2), 794S–797S.

Ouwehand, A.C., Tiihonen, K., Saarinen, M., Putaala, H., Rautonen, N., 2008. Influence of a combination of *Lactobacillus acidophilus* NCFM and lactitol on healthy elderly: intestinal and immune parameters. Br. J. Nutr. 18, 1–9.

Penders, J., Vink, C., Driessen, C., London, N., Thijs, C., Stobberingh, E.E., 2005. Quantification of *Bifidobacterium* spp., *Escherichia coli* and *Clostridium difficile* in faecal samples of breast-fed and formula-fed infants by real-time PCR. FEMS Microbiol. Lett. 243, 141–147.

Pryde, S.E., Duncan, S.H., Hold, G.L., Stewart, C.S., Flint, H.J., 2002. The microbiology of butyrate formation in the human colon. FEMS Microbiol. Lett. 217, 133–139.

Schabereiter-Gurtner, C., Pinar, G., Lubitz, W., Rolleke, S., 2001. An advanced molecular strategy to identify bacterial communities on art objects. J. Microbiol. Methods 45, 77–87.

Shreiner, A., Huffnagle, G.B., Noverr, M.C., 2008. The microflora hypothesis of allergic disease. Adv. Exp. Med. Biol. 635, 113–134.

Sokol, H., Seksik, P., Rigottier-Gois, L., Lay, C., Lepage, P., Podglajen, I., et al., 2006. Specificities of the fecal microbiota in inflammatory bowel disease. Inflamm. Bowel Dis. 12, 106–111.

Suau, A., Bonnet, R., Sutren, M., Godon, J.J., Gibson, G.R., Collins, M.D., Dore, J., 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl. Environ. Microbiol. 65, 4799–4807.

Tiihonen, K., Tynkkynen, S., Ouwehand, A., Ahlroos, T., Rautonen, N., 2008. The effect of ageing with and without non-steroidal anti-inflammatory drugs on gastrointestinal microbiology and immunology. Br. J. Nutr. 100, 130–137.

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., Gordon, J.I., 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444, 1027–1031.

Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R., Gordon, J.I., 2007. The human microbiome project. Nature 449, 804–810.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Dunan, A., Ley, R.E., et al., 2009. A core gut microbiome in obese and lean twins. Nature 457, 480–485.

Woodmansey, E.J., McMurdo, M.E., Macfarlane, G.T., Macfarlane, S., 2004. Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. Appl. Environ. Microbiol. 70 (10), 6113–6122.

Woodmansey, E.J., 2007. Intestinal bacteria and ageing. J. Appl. Microbiol. 102, 1178–1186.

Yu, Y., Lee, C., Kim, J., Hwang, S., 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol. Bioeng. 89 (6), 670–679.

Zhang, H., Dibaise, J.K., Zuccolo, A., Kudrna, D., Braidotti, M., Yu, Y., et al., 2009. Human Gut Microbiota in Obesity and After Gastric bypass. PNAS Early Edition January 21.

13.3. Poster presentation - International Probiotic Conference
June 2008, Slovakia

Effects of Ageing and Vegetarism on Diversity and Population sizes of Clostridium Cluster IV assessed with TaqMan PCR and PCR-DGGE fingerprinting

Liszt K., Zwielehner J., Hippe B., Haslberger A.G.
Department of Nutritional Sciences, University of Vienna, Austria.

Background & Objectives: Members of Clostridium cluster IV are involved in beneficial functions of the human colonic microbiota such as carbohydrate breakdown, butyrate production and a proposed role in chronic inflammation status of the intestine. 22% of faecal microbiota belongs to *Clostridium* cluster IV. We investigated effects on diet and ageing using qualitative and quantitiative methods.

Methods: Faecal microbiota of 15 institutionalized elderly, 15 young vegetarians and 14 omnivores were compared using quantitative TaqMan PCR and PCR-DGGE fingerprinting. For TaqMan PCR a probe was designed with CLC DNA workbench supplementary to previously published Clostridum cluster IV specific primers (Table 1). Relative quantitation analysis was used to calculate the relations of Clostridium cluster IV and bacteria. Reference systems for DGGE fingerprinting and TaqMan PCR were constructed from clone libraries. Diversity was assessed via PCR -DGGE fingerprinting, the calculation of Shannon's diversity index on the bandpattern data and cluster analysis.

Target bacterial group	Primer pair and Probe	sequence (5' - 3')	Size (bp)	Conc. [µM]	Reference
All bacteria	BAC-338-F	ACT CCT ACG GGA GGC AG	468	10	[1]
	BAC-805-R	GAC TAC CAG GGT ATC TAA TCC		10	[1]
	BAC-516-P	(FAM)-TGC CAG CAG CCG CGG TAA TAC-(BHQ-1)		2	[1]
Clostridium cluster IV	sg-Clep-F	GCA CAA GCA GTG GAG T	239	4	[2]
	sg-Clep-R	CTT CCT CCG TTT TGT CAA		4	[2]
	Clep-P**	(FAM)-AGG GTT GCG CTC GTT-(BHQ-1)		2	This study

** position of target site (numbering corresponding to *E. coli* 16S rRNA gene) 1082 to 1107.

Table 1: Primers and probes used in this study

Results: Significant reductions of *Clostridium* cluster IV were observed in elderly (1.3 fold; p= 0.039) and to a lesser extent in vegetarians (Fig.1). Cluster analysis and Jackknife test of PCR-DGGE fingerprinting showed a tendency for clustering for omnivores whereas vegetarians and elderly could not be grouped (Fig. 2, Fig. 3). Omnivores showed more common bands than vegetarians where some of them are present in nearly all omnivores but less present in vegetarians and elderly (Fig. 4). One sequence identified to match next to Faecalibacterium prausnitzii^T (similarity 96.7%) was prevalent in DGGE fingerprintings of 13 omnivores but only in 6 vegetarians and 4 elderly.

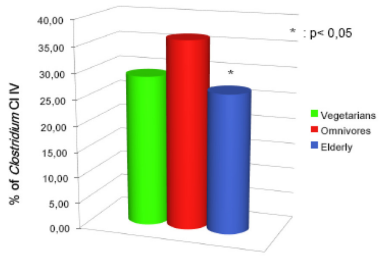


Figure 1: Relative quantification of Clostridium cluster IV with qrt-PCR and the primer and probe set of Table 1
Proportions of *Clostridium* cluster IV of total bacterial load.

Legend for all Figures:
Vegetarians:
Omnivores:
Elderly:

Figure 3: Jackknife (overall 54,76 %) of *Clostridium* Cl IV - DGGE-fingerprints

	28.57	0.00	7.14
	57.14	100.00	57.14
	14.29	0.00	35.71

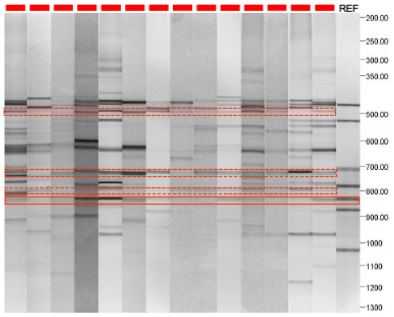


Figure 4: Band homogeneity of *Clostridium* cluster IV in omnivores demonstrated by DGGE-fingerprints of 16S rRNA coding regions with primer pair ClepF/ClepR-GC [3]
Marked bands are present in nearly all samples. Bands in the continuous red line match next to Faecalibacterium prausnitzii^T (similarity 96.7%).

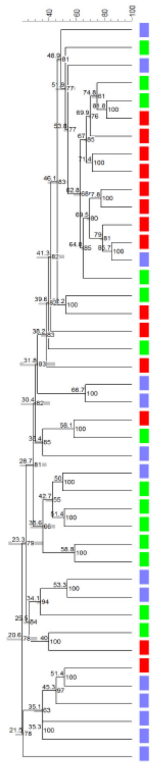


Figure 2: Cluster analysis of *Clostridium* Cl IV - DGGE-fingerprints (Dice coefficient). Error flags, similarity values and cophenetic correlations are shown in the tree.

Conclusions: The combination of quantitative rt-PCR and PCR-DGGE fingerprinting is a promising approach for monitoring individual characteristics as a possible basis for individualized pro-, pre- or synbiotic intervention. The present study shows quantitative and qualitative differences of *Clostridium* cluster IV between vegetarians and omnivores and a significant decrease in elderly.

References::
[1] Youngseob, Y., Changsoo, L, Jaai, K, Seokhwan H. 2005. Group-Specific Primer and Probe Sets to Detect Methanogenic Communities Using Quantitative Real-Time PolymeraseChain Reaction. Wiley InterScience.
[2] Matsuki, T., K. Watanabe, J. Fujimoto, T. Takada, and R. Tanaka. 2004. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. Applied and Environmental Microbiology 70:7220-7228.
[3] Shen J., Zhang B., Wei G., et al. 2006. Molecular Profiling of the *Clostridium leptum* Subgroup in Human Fecal Microflora by PCR-Denaturing Gradient Gel Electrophoresis and Clone Library Analysis

13.4. Poster presentation- 19th International conference of nutrition, October 2009, Bangkok

CHARACTERIZATION OF FAECAL MICROBIOTA IN VEGETARIANS COMPARED TO OMNIVORES USING QPCR AND PCR-DGGE FINGERPRINTING

Kathrin Liszt[1], Jutta Zwielehner[1], Berit Hippe[1], Cornelia Laßl[1], Roman Thaler[1], Alexander G. Haslberger[1]*.

Affiliation: [1]Department of Nutritional Sciences, University of Vienna, Austria

Background: The human body, especially the human intestinal tract harbors a large, active and very complex community of microbes, which contain 100-fold more genes than does the human genome [1]. Furthermore, these gut microbes have been associated with different diseases like colorectal cancer, inflammatory bowel diseases, obesity and atopic diseases [2-4]. The microbes as also those diseases have been shown to be influenced by diet. Therefore we investigated if vegetarian diet (e.g., little or no animal protein, low fat and high fibre content) may influence microbial subgroups of the intestinal microbiota.

Aims: The aim of this study was to investigate the quantitative and qualitative changes of *Bacteria*, *Bacteroides*, *Bifidobacteria* and *Clostridium* cluster IV in faecal microbiota associated with vegetarian diet.

Material and Methods: Faecal microbiota of 15 vegetarians and 14 omnivores were compared using quantitative PCR and PCR-DGGE fingerprinting. Bacterial subgroups were quantified as percentage of the total bacterial DNA. Bacterial diversity was assessed using PCA (principal components analysis).

Results

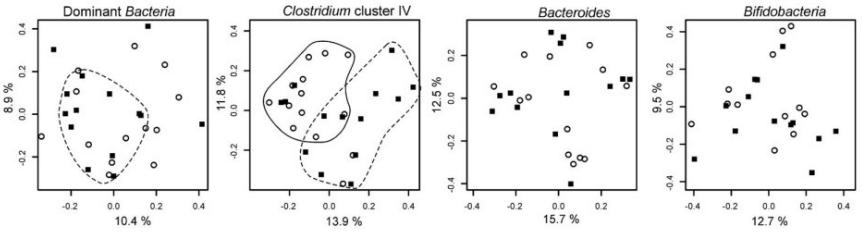
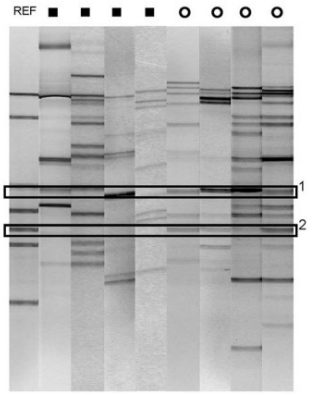


Figure 2: PCA of DGGE fingerprints of 16S rRNA coding regions of dominant *Bacteria*, *Clostridium* cluster IV, *Bacteroides* and *Bifidobacteria* in faecal samples.
○, omnivores; ■, vegetarians



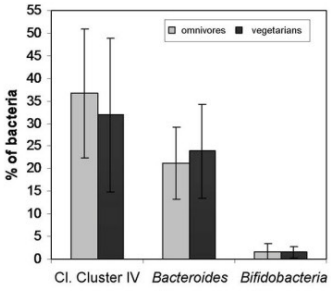


Figure 1: Proportions of *Bacteroides*, *Bifidobacteria* and *Clostridium* cluster IV of the total bacterial load in stool samples of vegetarians and omnivores.

Figure 3: PCR-DGGE bandpatterns of 16S rRNA coding regions of *Clostridium* cluster IV amplified with primer pair sg-Clept-F-GC/sg-Clept-R. Bands that were observed more frequently in omnivores than in vegetarians are indicated with a black box. 1: 94.7% similarity to *Clostridium* sp. BI-114^T and 97.9 % similarity to uncultured bacterium DQ793301; 2: 96.7 % similar to *Faecalibacterium prausnitzii*^{T} and 99.5 % to *Faecalibacterium prausnitzii* AJ270469 1: *Clostridium* sp. BI-114^T (similarity 94.7%) and uncultured bacterium DQ793301 (similarity 97.9 %).
○, omnivores; ■, vegetarians; REF, reference lane

Results:
Vegetarians had 19 % higher counts of bacterial DNA than omnivores.
Furthermore, we could see a tendency for lower counts in *Clostridium* cluster IV (31.86 ± 17.00 %; 36.64 ± 14.22 %) and higher counts in *Bacteroides* (23.93 % ± 10.35 %; 21.26 ± 8.05 %) (Figure 1).
PCA suggests a grouping of dominant bacteria and members of *Clostridium* cluster IV (Figure 2).
Two of 12.75 ± 3.37 bands per person of *Clostridium* cluster IV with 96.7% similarity to *Faecalibacterium prausnitzii*^{T} ($p<0.005$) and 94.7 % similarity to *Clostridium* sp. BI-114^T ($p<0.022$) appeared significantly more frequently in omnivores than in vegetarians (Figure 3).

Conclusions: Vegetarian diet seems to affect the intestinal microbiota, especially in changing the diversity of *Clostridium* cluster IV. Due to high interindividual deviations we observed a only a tendency for decreasing amounts of Clostridium cluster IV in vegetarians. It remains to be determined how these shifts might affect host metabolism and disease risks.

This work was supported by the Hochschuljubiläumsfond of the Austrian national bank.

Reference: [1] Gill, S. R., M. Pop, et al. (2006). "Metagenomic analysis of the human distal gut microbiome." Science (New York, N.Y.) 312(5778): 1355-1359.
[2] Li M, Wang B, Zhang M, Rantalainen M, Wang S, Zhou H, Zhang Y, Shen J, Pang X, Zhang M, Wei H, Chen Y, Lu H, Zuo J, Su M, Qiu Y, Jia W, Xiao C, Smith LM, Yang S, Holmes E, Tang H, Zhao G, Nicholson JK, Li L, Zhao L: Symbiotic gut microbes modulate human metabolic phenotypes. Proc Natl Acad Sci U S A 2008;105:2117-2122.
[3] Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmee E, Cousin B, Sulpice T, Chamontin B, Ferrieres J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, Burcelin R: Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007;56:1761-1772.
[4] Penders J, Thijs C, van den Brandt PA, Kummeling I, Snijders B, Stelma F, Adams H, van Ree R, Stobberingh EE: Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study. Gut 2007;56:661-667.

14. CURRICULUM VITAE

Name	Kathrin Ingrid Liszt
Date of birth	01/02/1985
Place of birth	Oberwart in Burgenland

Education

Since 10/2003	Student of Nutritional Sciences at the Department of Life Sciences at University of Vienna
06/2003	Matura –university entrance diploma

Practical experiences

Diploma thesis 2007/09	Quantitative and qualitative analysis of Clostridium cluster IV in faeces with molecular methods with Univ.Doz.Dr. Alexander Haslberger
June 2008	Poster presentation at the International Probiotics Conference in Slovakia
August 2008	Internship by Edith Kubiena (dietician)
February 2007	Internship in the working group of Univ.Doz.Dr. Alexander Haslberger,
Sept 2006	Internship at the Department of Nutritional Sciences at the University of Vienna, elevation of the nutritional status
Since february 2006	Temporary employment at the doctor's secretary at the hospital "Krankenhaus der Barmherzigen Schwestern" in Vienna

Publications:

Kathrin Liszt, Jutta Zwielehner, Michael Handschur, Berit Hippe, Roman Thaler, Alexander G. Haslberger; Characterization of *Bacteria*, *Clostridia*, *Bacteroides* in faeces of vegetarians using qPCR and PCR-DGGE fingerprinting; Ann Nutr Metab. 2009 Jul 27;54(4):253-257.

Zwielehner, J., Liszt K., Handschur M., Lapin A., Haslberger A.; Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of Bacteroides, bifidobacteria and Clostridium cluster IV in institutionalized elderly. Exp Gerontol. 2009 Jun-Jul;44(6-7):440-6. Epub 2009 Apr 17.

Poster

Liszt K, Zwielehner J, Hippe B, Laßl C, Thaler R, Haslberger AG 2009, Characterization of faecal microbiota in vegetarians compared to omnivores using qPCR and PCR-DGGE fingerprinting. 19th International congress of Nutrition (ICN October 2009), Bangkok

Liszt K, Hippe B, Zwielehner J, Haslberger AG 2008, Effects of ageing and vegetarism on diversity and population sizes of Clostridium cluster IV assessed with TaqMan-PCR and PCR-DGGE fingerprinting. International Probiotic Conference June 2008, Slovakia

Zwielehner, J., Handschur M., Reid S., Liszt K., Thaler R., Hippe B., Haslberger A.G.; Combined PCR-DGGE and quantitative-PCR analysis for the assessment of immune-relevant shifts of the GI-microbiota.

Handschur M, Zwielehner J, Irez S, Liszt K, Thaler R, Haslberger AG: Analysis of selected groups of bacteria in faeces of consumers adhering to different diets using combined DGGE analysis and quantitative TaqMan- PCR. Abstracted in Cibus Vol. 3, No. 2, Aug 2007: 37.