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DISSERTATION

Titel der Dissertation

Molecular analysis of intestinal microbiota composition and function in individuals with different diets, age and disease status.

PCR-DGGE fingerprinting, qPCR and stable isotope probing applied to microbiota samples.

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2 Introduction: the human gut microbiota.

2.1 Microbiota composition

Establishment of the intestinal microbiota has been shown to be a progressive process [1]. This process of increasing diversity is required for proper development and is important for overall health. The major functions attributed to the microbiota present in the gut begin to manifest at the end of the second year of life and comprise: i) nutrients absorption and food fermentation [2], ii) stimulation of the host immune system [3] and iii) barrier effects against pathogens [4]. Once climax composition is achieved near the end of adolescence, this ecosystem displays a high stability in healthy adults [5].

In adults, *Bacteroidetes* and *Firmicutes* are the most prevalent phyla present, the latter of which combines the values obtained for the dominant *C. leptum* and *C. coccoides* groups and the sub-dominant *Lactobacillus* group [6]. The *Bifidobacterium* genus is present in eight to ten-fold lower numbers than the two major phyla. *E. coli* was found to be present at 7.7 log10 CFU/g, also consistent with its characteristic sub-dominant population in adults [7] where this species is considered a dominant species in infants. Members of the *Actinobacteria* phylum such as bifidobacteria are minor constituents of the microbiota with tremendous health effect to the host. Bifidobacteria enhance the barrier function of the gut epithelium [8]. Low abundance of bifidobacteria has been associated with LPS-induced endotoxaemia, a chronic low-grade inflammation [9]. Recent studies indicated that modifications occur in the microbiota composition in elderly individuals. For example, a reduction in the numbers of *Bifidobacteria* and *Bacteroides* has been observed, accompanied also by a decrease of *Lactobacilli*. A commensurate increase in the number of facultative anaerobes also highlights the variation between adults and elderly individuals [10, 11, 12, 13].

The Firmicutes are by far the most diverse phylum in the human GI tract. Members of Firmicutes are involved in fermentation of non-digestible carbohydrates and production of short chain fatty acids (SCFAs). Short chain fatty acids are intermediates and end products of microbial breakdown of polysaccharides. In the GI tract the following SCFAs are prevalent: formate, acetate, propionate, butyrate, isobutyrate, valerate, isovalerate and caproic acid. Formate is likely to be used as substrate in the one-carbon-metabolism of intestinal bacteria [14]. Acetate and propionate serve for gluconeogenesis in the liver [15]. Butyrate is the major energy source of colonocytes and stimulates proliferation in healthy colonic epithelial cells [16, 17]. In colonic tumor butyrate is known to inhibit proliferation in cells and cell lines [18, 19]. One explanation for the butyrate paradox is that healthy cells have an efficient butyrate metabolism resulting in low intracellular butyrate concentrations and therefore a decrease in capacity to inhibit growth [20]. In colon cancer cell lines, β-oxidation and mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS2) expression are impaired [20]. HMGCS2 converts acetyl-CoA to ketone bodies [21] thereby preventing the accumulation of acetyl-CoA [22]. The decreased oxidation rate of butyrate may result in increased intra-cellular butyrate concentrations in tumor cells, hence causing increased histone deacetylation and subsequently decreased proliferation [23]. Some of the most prominent butyrate producers are Firmicutes: the *Clostridium* clusters *IV* and *XIV*, among which the *Roseburia* spp. and *Eubacterium* spp. have drawn a lot of attention recently for their high butyrate production rate [24].

Commensal bacteria promote angiogenesis and development of the intestinal epithelium and have been shown to be essential for normal development and function of the immune system [25].

It has been suggested that every human possesses an individual core microbiota of some species that is resilient to modifications throughout the life span [26]. The hypothesis of a global core microbiota that all humans share has been cast into doubt. Recent results suggest that there is a core microbiome [27], a collection of microbial genes that all human beings have in common. Such a core microbiome comprises

genes rather than organismal lineages. Metabolic reconstruction of the ‘core’ microbiome revealed significant enrichment for a number of expected functional categories, including those involved in transcription and translation [27]. Metabolic profile-based clustering indicated that the representation of the ‘core’ functional groups was highly consistent across samples, and includes a number of pathways likely important for life in the gut, such as those for carbohydrate and amino acid metabolism (fructose/mannose metabolism, aminosugar metabolism, and N-glycan degradation) [27].

Kurokawa et al. described 2007 that the gene repertoires in the gut microbiomes are more variable and functionally less redundant in infants than in adults and children. All human gut microbiomes showed a significant over-representation of COGs (Cluster of Orthologous Groups of proteins) classified into ‘carbohydrate transport and metabolism’ and an under-representation of COGs classified into ‘lipid transport and metabolism’ [14]. In adults and in children COGs classified into ‘defence mechanisms’ were over-represented. The clusters ‘cell motility’, ‘secondary metabolite biosynthesis, transport and catabolism’ and ‘post-translational modification, protein turnover, chaperones’ were minor gene contents in the microbiomes assessed [14]. The low abundance of flagella in intestinal microbes is likely to be an adaptation to the human gut environment where i) the continuous movement of the luminal contents by peristalsis make bacterial cell motility reduandant and ii) flagellated microorganisms are effectively recognized by toll like receptor 5 and eliminated by the host immune cells. The microbiota of obese individuals have been found to be enriched in genes for energy harvest [27].

2.2 Host-microbe interactions I: how the gut microbiota interacts with the human immune system.

The gastro-intestinal epithelium is the first line of the defence system against microbial pathogens such as Gram-positive and Gram-negative bacteria, fungi and viruses. Innate immune cells such as macrophages and DCs (dendritic cells) directly kill the pathogenic micro-organims through phagocytosis or induce the production of cytokines, which aid elimination of the pathogens [28, 29, 30]. Immunity against microbial pathogens and tolerance against commensals primarily depends on the recognition of pathogen components by innate receptors expressed on immune and non-immune cells. Innate receptors are evolutionarily conserved germ-line-encoded proteins and include TLRs (Toll-like receptors), RLRs [RIG-I (retinoic acid-inducible gene-I)-like receptors] and NLRs (Nod-like receptors). These families of receptors are collectively known as PRRs (pattern-recognition receptors) [31], which recognize the specific molecular structures of pathogens known as PAMPs (pathogen-associated molecular patterns) in various compartments of cells such as the plasma membrane the endolysosome and the cytoplasm. PRRs recognize pathogens or pathogen-derived products and components thereof in different cellular compartments such as the plasma membrane, the endosomes or the cytoplasm, and induce the expression of cytokines, chemokines and co-stimulatory molecules to eliminate pathogens and instruct pathogen-specific adaptive immune responses [32]. The adaptive immune system consists of B- and T-cells, which provide pathogen specific immunity to the host and –in absence of atopic disease- tolerance towards beneficial and commensal microorganisms of the GI tract. B- and T-cells are shaped through somatic rearrangement of antigen receptor genes. B-cells produce pathogen-specific antibodies to neutralise toxins produced by pathogens, whereas T-cells provide the cytokine milieu to clear pathogen-infected cells through their cytotoxic effects or via signals to B-cells [33].

According to the hygiene theory atopic diseases and autoimmune diseases are caused by a disturbed ratio of T_{h1}, T_{h2} and T_{reg} cells.

Regulatory T Cells (T_{reg}) excrete TGF-β and IL-10, two anti-inflammatory cytokines that are able to suppress the activation of T_{h1} and T_{h2} cells. Th_{17} T cells have recently been described as third subset of T helper cells induced by IL-1β, IL-6 and TGFβ [34]. Secretion of IL-23 is thought to be important for the maintenance of Th_{17} cells found to be associated with autoimmune diseases such as multiple sclerosis and rheumatoid arthritis [35]. Macrophages are capable of excreting IL-10 too, thus suppressing pro-inflammatory T_{h1} and T_{h2} cells as well. Epithelial cells and dendritic cells possess PRRs that are able to sample bacterial antigens from the gut lumen. Dendritic cells project their dendrites through the epithelial layer to sample the intestinal contents and subsequently migrate to afferent lymph nodes to present their cargo to T cells [36]. Macrophages dwelling in the lamina propria form a unique cell subset that fails to generate pro-inflammatory responses to TLR ligands and suppresses Th_{17} T cell priming by neighbouring dendritic cells [37]. This shows that also dynamic interactions of antigen presenting cell (APC) subsets residing in the lamina propria may play a role in effector T cell differentiation and polarization after (commensal) antigen recognition. Only dendritic cells are capable of inducing T_{regs}, which are immunesuppressive and regulate T_{h1}/T_{h2} balance [38]. The differential ability to induce inflammatory responses of macrophages and dendritic cells may be attributable to different requirements for responsiveness to steady-state vs. inflammatory antigens for priming of T cells.

In addition to dendritic cells- mediated antigen transport into the mesenteric lymph nodes there seem to be additional unknown mechanisms: In mice whose dendritic cells are deficient in CC chemokine receptor 7 the capsular polysaccharide from the commensal *Bacteroides fragilis* corrects systemic T cell deficiency and primes the splenic T_{h1} response of orally challenged or colonized mice [39]. Mucosal dendritic cells are considered more tolerogenic than other dendritic cells. Their expression patterns of TLRs are unique and characteristic for the microenvironment they

regularly encounter. Mucosal DCs from the lamina propria, Peyer's patches, mesenteric lymph nodes and the lung have a property to induce T_{H2} responses in *in vitro* T cell priming assays and to induce the expression of IL-10 and possibly TGF-β. Mucosal DCs may be specialized in inducing a non-inflammatory environment and in providing help to B cells via the activation of T_{H2} cells [40]. This is consistent with the fact that many 'tolerogenic' responses to mucosal antigens, for example, to commensal organisms, are associated with the generation of antibody responses [41] rather than with a broad immunological unresponsiveness [40].

2.3 Host-microbe interactions II: examples of how the gut microbiota contributes to the human metabolism.

In spite of tremendous progress in research the interplay between microbiota composition and microbiome functions is still limited. Recent work points out that the human gastro-intestinal microbiota must be understood as a microbial organ whose metabolism provide essential functions to the host rather than just supplementary nutrient acquisition.

Phenolic compounds have been described to have beneficial health effects related to their anitoxidant, anti-inflammatory, antiestrogenic, cardioproective, cancer chemopreventive and neutoprotective properties [42]. Most dietary polyphenols are transformed in the colon by the intestinal microbiota before absorption. This conversion is often essential for absorption and modulates the biological activiy of these dietary compounds. Therefore the colon has to be considered as an active site for metabolism rather than a simple excretion route [42]. Microbial metabolites are absorbed from the colon after deconjugation and transformed by the human cell enzymes into phase II conjugates including methyl ether glucuronides and sulfates metabolized in the liver, resulting in their glucuronidated and sulfated derivatives [43]. Through enterohepatic recirculation, conjugated compounds are excreted by the liver as components of bile into the intestine, and the deconjugated compounds are regenerated by microbial enzymes before being reabsorbed. Onions, apples, broccoli,

tea, and red wine are rich in flavonols and are the main dietary sources of these compounds in Western populations [44]. Almost all soy isoflavones exist as glucosides, which are not absorbed intact across the enterocytes of healthy adults because of their hydrophilic properties and molecular weights. The isoflavon glucosides are very water-soluble. Their bioavailability requires the conversion of glucosides into the principal bioactive aglycones (daidyein, genistein and glycitein) via the action of intestinal β-glucosidase from bacteria that colonize the small intestine for uptake to the peripheral circulation [45]. The aglycones are either absorbed intact or further metabolized by intestinal microbiota [46]. Genistein is converted to *p*-ethylphenol and 4-hydroxyphenyl-2-propionic acid, whereas daidzein is reduced to *O*-demethylangolensin (*O*-DMA) and equol [47]. Humans that consume dadzein produce relatively low amounts of the highly antioxidant equol as a consequence of specific compounds in the intestinal microbiota [47]. Only approximately one third to half of the population is able to metabolize dadzein to equol [47]. *Bacteroides ovatus, Streptococcus intermedius, Ruminococcus productus, Enteroccuse faecium* EPI1, *Lactobacillus mucosae* EPI2, *Finegoldia magna* EPI3 and *Veillonella* sp. EP have been demonstrated to be capable of this conversion [48]. Bacteria that possess *β*-glucosidase enabling them to hydrolyse glycosides are *Streptococcus faecalis, Eubacterium rectale, Clostridium spenoides, C. saccharogumia, C. cocleatum, Bacteroides ovatus, B. fragilis and B. distasonis* [48].

2.4 Molecular methods for the assessment of the human microbiota.

Classical microbiological methods depend on culturing techniques and have been applied to various ecosystems including the human gastro-intestinal microbiota. Comparison of those results with modern molecular techniques allows estimating that with culturing only 20-40% of the microbiota diversity can be targeted [49]. The isolation of microbiota constituents allows analyzing their phenotype and metabolic capacities and behaviour in coculture with defined microorganisms. Coculture studies

show that many ecological niches such as living of short chain fatty acids can only be filled by syntrophic consortia of different microbes [50] [51]. Coculture of the xylanolytic *Roseburia intestinalis* with H_2-utilizing *Ruminococcus hydrogenotrophicus* dramatically increased butyrate production [52].

Molecuar methods, many of which are based on the polymerase chain reaction, overcome the limitations due to the fastidious nature of many gut microbes.

2.4.1 Polymerase chain reaction (PCR)

PCR was developed by Kary Mullis and firstly published in 1985 [53]. It is an *in vitro* method for DNA amplification using a polymerase from thermophilic microbes such as the *Thermus aquaticus* from which the frequently used *Taq* polymerase is derived. A region of dsDNA is amplified using two primers, a polymerase, dNTPs and a buffer containing stabilizing salts and $MgCl_2$. The introduction of PCR in microbial ecology of the human gut revolutionized our knowledge about this ecosystem. Culturing studies have overestimated the prevalence of coliform bacteria such as the *Proteobacterium E.coli* in the human gut. Molecular analyses showed that facultative anaerobic bacteria were only a minority within the colon (0.1%) and that strictly anaerobic microbes were by far the most dominant group of microorganisms [54].

PCR bias

All PCR-based methods are affected by bias. Biases are introduced by different primer binding energy, or by rehybridisation of PCR products so that species evenness in PCR products appears to be even, even if the initial species abundances were not so at all [55]. The formation of heteroduplexes can be problematic in downstream applications such as cloning and sequencing [56]. Another risk PCR reactions bear is the formation of chimera through incompletely extended primers and template switching [57].

Limiting the number of PCR cycles and choosing reasonable PCR conditions that allow for complete elongation of primers can avoid all the biases mentioned above.

Biases are furthermore introduced through incomplete lysis of bacteria during DNA extraction and differential affinity of PCR primers. Hong et al estimate that a single DNA extraction and primer combination recovers only half of the richness/species diversity that can be recovered using three such combinations [58]. Applied on environmental samples from anoxic waters, multiple PCR primer sets retrieved novel lineages that escaped the single PCR primer approach. Surprisingly there was even hardly any overlap between datasets retrieved from different primer sets on microeukariotic diversity in anoxic waters [58].

Quantitative PCR (q-PCR)

In real-time or quantitative PCR a targeted DNA molecule is simultaneously amplified and quantified. Two common methods for detection of products in q-PCR are the use of fluorescent dyes that intercalate with ds DNA fragment and the use of fluorescently labelled oligonucleotides.

Fluorescent dyes such as SybrGreen or ethidiumbromide intercalate with ds DNA in a non-specific manner. The increase in PCR product is measured at the end of each elongation step after excitation with a laser. The generated DNA fragments can be further analyzed after cycling in a melt curve analysis wereby the temperature is gradually increased until the PCR products melt and the fluorescence decreases. Primer dimers, different PCR products as well as unspecific binding can be distinguished from each other. Given that PCR products of different strains melt at different temperatures their relative abundances can be estimated according to their peak area in melt curve analysis. Quantification using fluorescent dyes is easy to apply, but can be affected by several sources of bias such as unspecific binding or the formation of primer dimers as these fragments equally contribute to increase in florescence.

Different approaches for real-time quantification with fluorescently labelled oligonucleotides have been developed to specifically detect a fragment of interest. In multiplex q-PCR different amplicons can be simultaneously detected via use of different probes that are labelled with fluorophors emitting at different wavelengths. Multiplex PCR has been applied to differentiate the phylogenetic groups and virulence genes in *Enterobacteriaceae* isolates from children with coeliac disease [59]. The authors elegantly demonstrated that celiac disease was associated with higher prevalence of virulence factors P fimbriae (papC), capsule K5 (sfaD/E) and haemolysin (hlyA) gene at lower diversity within *Enterobacteriaceae.*

2.4.2 Molecular fingerprinting techniques

In contrast to cloning and sequencing, fingerprinting techniques enable the rapid analysis of large sample numbers including replicates. They enable quantitative or semi-quantitative analysis of relative abundances of different community members [60]. The analysis of the microbiota of epidemiologically defined groups allows an estimation of microbial population dynamics in response to epidemiological variables such as e.g. diet, age and medication.

Denaturing gradient gel electrophoresis (DGGE) is a form of electrophoresis where nucleic acids migrate in a chemical gradient according to their GC-content. A similar method is TGGE (temperature gradient gel electrophoresis) where nucleic acids are separated in a temperature gradient.

Leonard Lerman invented DGGE in 1979 [61] and applied it to detect single base substitutions in genomic DNA [62]. Muyzer [63] introduced DGGE to analyze the genetic diversity of complex microbial populations. Alternative fingerprinting methods are terminal restriction length polymorphism (T-RFLP) [64] and single strand conformation polymorphism analysis (SSCP) [64, 65]. The interpretation of microbial fingerprinting techniques is very complex due to the vast diversity of microbial gut

communities. A number of methods have been used for quantitative analysis of fingerprint patterns and comparison of communities [66]. Statistical models applied to study plant and animal distributions in population ecology are gaining relevance among microbial ecologists, but the adaptation of such methods is still under development and these are mostly used for exploratory analysis of molecular data [67]. All methods suffer from a number of unavoidable limitations including bias resulting from samples run on different gels or machines; PCR bias or low quality amplification products; differences in detection limits, which affect the interpretation of “false” and “true” bands or peaks; and lack of distinction between closely adjacent bands or peaks, particularly when these differ significantly in intensity or size [68, 69].

A crucial step in the computational analysis of fingerprinting techniques is the digitalization of visual fingerprinting data. Variable staining of different gels is unavoidable and can be normalized via transforming fingerprinting data into functions. Then individual bands are expressed relative to the total lane intensities [68]. Bias that arises from comparison of different gels can be minimized by loading an artificial bandpattern in triplicate on each gel as a marker [68]. Closely adjacent bands or peaks forming a ‘shoulder’ are often hard to resolve correctly. Software developed for the digitalization of fingerprinting data such as GelComparII (www.applied-maths.com) allows changing settings to find the best approximation for correct band definition. This trial-and-error approach and the necessity to inspect and adjust the assigned bands manually is clearly a weak point in fingerprinting analysis. It is quite likely that different investigators end up with slightly different band comparison datasets.

2.4.3 Multivariate statistical methods for the analysis of microbial population dynamics.

Computational tools such as (functional) principal components analysis (PCA and FPCA), clustering of correlations after Dice or Pearson, self organising maps (SOM) or partial least squares regression (PLS-R) have been found to be useful in the interpretation of fingerprinting datasets.

principal components analysis (PCA)

Principal component analysis (PCA) was developed in 1901 by Karl Pearson.

PCA is a bilinear modelling method that gives an interpretable overview of the main information in a multidimensional data table. The information carried by the original variables is projected onto a smaller number of underlying (latent) variables called principal components. The first principal component covers as much of the variation in the data as possible. The second principal component is orthogonal to the first and covers as much of the remaining variation as possible, and so on. By plotting the principal components, one can view relationships between different variables and detect and interpret sample patterns, groupings, similarities, or differences [70]. PCA is used as a tool in explorative data analysis and for making predictive models. Dollhopf et al [71] were the first to publish the application of PCA ordination on community fingerprinting. The disadvantage of PCA becomes apparent in noisy datasets that contain smear and differential special clearness. Although a valid method for the analysis of population dynamics PCA produces a considerable number of misclassifications. The difficulty that PCA has in clearly resolving underlying variables within a dataset becomes apparent when considering that typically only about 30% of variation can be explained with the first two principal components.

functional principal components analysis (FPCA)

FPCA regards band intensities as a mathematical function of the location on the gel and explicitly includes neighbourhood structure in the analysis [60]. In experimental datasets generated from standardized soil samples, FPCA generated about only halve as many misclassifications as the classical PCA approach. Furthermore, FPCA was able to extract about 58.4% of variation with the first two principal components from the dataset, where PCA could only explain 31% of the variation. In FPCA [60] the scores of the individual samples on the principal components are basis for hierarchical cluster analysis using Ward's method [60].

partial least squares analysis (PLS)

Partial Least Squares regression (PLS) is based on linear transition from a large number of original descriptors to a new variable space based on *small number of orthogonal factors (latent variables)*. In other words, factors are mutually independent (orthogonal) linear combinations of original descriptors. Unlike some similar approaches (e.g. principal component regression PCR), latent variables are chosen in a way to provide maximum correlation with dependent variable; thus, PLS model contains the smallest necessary number of factors [60]. PLS regression technique is especially useful in quite common cases where the number of descriptors (independent variables) is comparable to or greater than the number of compounds (data points) and/or there exist other factors leading to correlations between variables. In this case the solution of classical least squares problem does not exist or is unstable and unreliable. On the other hand, PLS approach leads to *stable*, correct and highly predictive models even for correlated descriptors [60]. PLS has been applied by Furet et al. 2009 to cluster the normalized results of a qPCR analysis of human and farm animal microbiota. The authors found a clear separation between human microbiota and the gut microbes of all farm animal assessed. They could

furthermore identify *Enterococcus* spp., the *Blautia coccoies* cluster and *Staphylococcus salivarius* as responsible for this clear separation [72].

self organizing maps neural networks (SOM)

Artificial neural networks (ANNs) may provide an alternative to conventional statistical methods because they detect nonlinear relationships, allow the visualization of complex data, and remain robust despite experimental variation. Phylogenetic reconstruction, classification of proteins, and genomic analysis are only a few applications of SOMs in molecular biology [71, 73, 74, 75]. Ecological applications for SOMs are also being explored for classification and modeling of populations and ecosystems [76, 77]. Recently, the application of SOM was demonstrated on microbiota composition data generated with the phylogenetic chip-platform HIT-CHIP (oral presentation, Probiotics, Prebiotics & New Food, 13-15th sept 2009, rome, italy).

2.4.4 linking phylogeny with metabolism

PCR based molecular methods retrieve nucleotide sequences, which then are compared to previously described, cultured species. This approach allows only an approximate guess on the specific function of the particular microbes prevalent in an ecosystem. Several molecular methods have been developed that allow insight into specific functions of also uncultured microorganisms and biofilms.

Fluorescence in situ hybridization (FISH)

FISH is a cytogenetic technique that uses fluorescent nucleotide probes to detect and localize specific DNA- or RNA sequences. FISH has been applied to diverse environmental samples such as activated sludge [78] and the human microbiota [79].

In contrast to PCR-based methods, where the spatial structure of the sample is disrupted in DNA/RNA extraction, FISH can be applied to resolve the spatial distribution of microorganisms in a biofilm. When fluorescent probes specific for a functional group of bacteria such as ammonia oxidizers are used, the comparison of probe-based enumeration with nitrification rates also provides a basis on which to measure the specific *in situ* activity per single cell [80]. Application of FISH to intestinal biopsies showed the spatial distribution of bacteria in the gut lumen and the mucus layer [81]. Van der Waaij et al did not observe any direct contact to the gut epithelium; bacteria were only detected at the luminal side of the mucus layer [81]. Bacteria in the transition zone from faeces to mucus: Fecomucus bacteria related to *Faecalibacterium prausnitzii* were mainly located in faeces; however, they can enter mucus in low concentrations. Mucophob bacteria were found to be related to bifidobacteria and avoid mucus and often even the edge of faeces. Mucotrop bacteria related to the *Helicobacter* genus prevail in the transition zone between faeces and mucus and may be completely absent in faeces [82].

Probing for answers with stable isotopes

Biomarker labelling

Several culture-independent approaches to linking microbial community function with the genetic identity of key organisms have begun to emerge. Stable-carbon-isotope labeling of cell wall lipid as biomarkers has been applied to study aquatic sediments [83]. The authors fed 13C-acetate to estuarine and brackish sediment samples and did not find incorporation of heavy label in the 17-carbon compounds polar-lipid-derived fatty acids (PLFAs) that have been proposed as specific biomarkers for Gram-negative bacteria. From this they conclude that the acetate reduction-coupled sulfate reduction in the marine sediments studied is mediated by the Gram-positive *Desufomaculum acetoxidans* and not by the more widely studied Gram-negative *Desufobacter* spp. A very clear knowledge of well studied biomarkers was a prerequisite for their heavy-

carbon-labeling experiments. In situ PLFA profiles are likely to have been generated by more than one organism. This complexity cannot be resolved using PLFAs as biomarkers.

When less is known about the microbial functions RNA or DNA are more elegant molecules to assess because unspecific primers allow unambiguously targeting the majority of microbial RNA varieties, especially when using a variety of different primer combinations as previously suggested [84].

FISH-MAR (fluorencence in sito hybridisation & microautoradiography)

Lee et al. combined FISH with microautoradiography in order to combine phylogenetic information with radioactive labeling [85]. Fluorescently labelled microorganisms and silver grains could be simultaneously observed at a high resolution by inverse confocal laser scanning microscopy.

FISH-MAR can be used to investigate in great detail the ecophysiology of defined organisms of interest, for which a FISH probe is available or can be designed. FISH-MAR is also suitable to hunt for and to quantify those microorganisms that are responsible for a certain physiological process.

Probes used for FISH only target small regions of the RNA. This may restrict their specificity and make taxonomic identification difficult- especially in environments such as the human GI tract that contain a large fraction of previously uncultured and undescribed species with strictly anaerobic and symbiotic growth requirements. Furthermore the number of bacterial populations that can be simultaneously identified in a single FISH experiment is limited by availability of different fluorophores, of which only seven have been thus-far identified. FISH-MAR does not measure total uptake of radiolabelled substrates but only assimilation into macromolecules [86]. Unincorporated labelled compounds are not retained within the cells and the detection of labelled macromolecules is not limited to nucleic acids. This being the case FISH-MAR is a very sensitive method that requires short incubation

times and thus can reduce the risk of false-positive results due to metabolic cross-feeding.

nucleotide stable isototope probing (SIP)

Stable-isotope labelled DNA and RNA can be recovered from total community nucleic acid extractions on the basis of its increased buoyant density and can be used as alternative, unambiguous biomarkers [87]. When using DNA as a biomarker, the rate of DNA synthesis in situ plays a pivotal role. DNA synthesis reflects organism replication rate and limits the enrichment of this biomarker. RNA synthesis rates are higher than those of DNA and are a reflection of cellular activity independent of replication. Ribosomal RNA constitutes the ribosome whereby which a cell is capable of shaping its environment. DNA/RNA SIP allows the retrieval of the full 16S ribosomal nucleic acid sequences from enriched clone libraries. Comparison with the extensive NCBI database for ribosomal RNA and DNA allows the identification of specific strains involved in the specific assimilatory process studied. Another advantage of nucleotide-stable isotope probing is that it provides access to the complete genome in a labelled nucleotide sample. Thus functional genes can be analyzed and previously unknown metabolic pathways can be elucidated.

Several limitations might render it difficult to prove that the heavy label has been consumed.

Dilution of the labelled substrate before assimilation and incorporation will dilute the proportion of ^{13}C that is incorporated into DNA/RNA. In simultaneous presence of ^{12}C substrate less DNA/RNA will be isotopically labelled and the analysis of primary consumers will become more difficult. Unlabelled carbon sources may include other organic compounds or the same substrate produced by internal processes in the environment.

The fate of the ^{13}C labelled atom of the heavy substrate can introduce considerable bias in stable isotope probing. Microbial metabolism of the heavy substrate may also

lead to the incorporation of ^{13}C into products and intermediates of metabolism, which might be assimilated by other, non-primary, organisms [88]. Production of the corresponding ^{12}C-labelled compounds and trophic interactions would dilute these modified substrates in a complex environment such as the human GI tract. It is thus very important to keep incubation times with ^{13}C-label short in order to avoid false-positive results due to metabolic cross-feeding and dilution of the label by endogenously generated substrate.

Manefield et al. [87] measured the $^{13}C/^{12}C$ ratio in an isotope ratio mass spectrometry (IRMS) analysis. For this analysis a minimal amount of 25µg carbon is required. To reach this bottom limit for IRMS analysis 1µg of DNA/RNA (0.36µg C) was cut with 61.6 µg of glucose (24.64 µg of C). In spite of this dilution, enrichment of ^{13}C in nucleic acid samples from a phenol-degrading bioreactor could be seen above natural levels (1.11% of all carbon). Members of the *Thauera* genus could be proved to be involved in phenol degradation in an aerobic industrial bioreactor. The stunningly sensitive IRMS analysis is promising for future application of SIP in linking phylogenetic identity with function in human gut microbiota science.

The inability of stable isotope probing to resolve trophic transfer renders SIP almost impossible for *in vivo* experiments. On its route through the GI system the degree of trophic transfers between constituents of the microbiota would lead to a dispersal of ^{13}C label into virtually all microbes present.

Thus SIP experiments targeting gut bacteria rely on *in vitro* experiments with faecal inoculum. Using an *in vitro* model system for the human intestine lactic acid bacteria and *Clostridium perfringens* could be shown to be the primary glucose-fermenting bacteria of the small intestine [89]. *Streptococcus bovis* and *C.perfringens* were found to be the most active glucose-fermenters. NMR analysis furthermore showed that lactate, acetate, butyrate and formate were the principal fermentation products [89] with significant enrichment of ^{13}C label. After modification of the model system to resemble the colonic microbiota bacteria related to *Ruminococcus bromii, Prevotella* spp., and *Eubacterium rectale* could be shown to be involved in starch metabolism

[90]. Integration of molecular and metabolite data suggested metabolic cross-feeding in the system where populations related to *Ruminococcus bromii* were the primary starch degraders. Strains related to *Prevotella* spp., *Bifidobacterium adolescentis* and *Eubacterium rectale* were suggested to be further involved in the trophic chain [90].

2.5 Aim of the thesis

In the light of the rapidly changing knowledge on the human microbiota, is appears clearly necessary to combine existing molecular methods and multivariate statistical tools in order to gain meaningful information from this highly diverse and intertwined habitat. The aim of this thesis was to apply PCR-DGGE fingerprinting, qPCR, functional gene analysis and stable isotope probing on faecal samples of epidemiologically defined groups of individuals such as those following different diets, elderly citizens and individuals with different pathological conditions. Furthermore, statistical methods should be explored for the meaningful interpretation of results.

3 Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in faecal population sizes and diversity of *Bacteroides*, bifidobacteria and clostridium cluster IV in institutionalized elderly.

3.1 Introduction

With ageing, a decrease in beneficial organisms such as *Lactobacilli* and *Bifidobacteria*, amongst other anaerobes, and an increase in the number of facultative anaerobes [91] 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* [63]. 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 [13, 92]. 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 [93]. 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 [92].

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 faeces are representative of inter-individual differences [94].

The aim of this work was to investigate shifts in GI microbiota associated with aging, 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.

3.2 Material and methods

3.2.1 study participants

Seventeen institutionalized elderly aged 86 ± 8 years, BMI 21.75 $\pm$ 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 $\pm$ 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.

3.2.2 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 sec 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, *Bacteroides thetaiotaomicron* DSM 2079^T, *Bifidobacterium longum* DSM20219^T, *B.longum* DSM 20211, *B.pseudolongum* DSM 20099 and *B.thermophilus* DSM 20210 were anaerobically cultivated on blood agar, *E.coli* IMBH 252/07 and clones were 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 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.

3.2.3 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 1.5mM $MgCl_2$. Bovine serum albumin (10mg/ml, Fermentas) was added to a final concentration of 400μg/ml, primer concentration in the reaction volume was 0.5μM. Amplifications were carried out in a Robocycler (Stratagene).

3.2.4 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 [95] and sg-Clep-R [96], which are specific for members of the *Clostridium* cluster IV, and 32f and 708r [97], 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 [68]. Clone inserts were sequenced by 'DNA confidence' (Vienna). Nucleotide sequences were corrected for vector and primer sequences in CodonCode 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/).

3.2.5 DGGE (denaturing gradient gel electrophoresis)

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 analyse samples for patterns in a) total *Bacteria*, b) *Bifidobacteria*, c) *Clostridium* cluster IV and d) *Bacteroides*. DGGE gels were prepared as described previously [98] 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 faecal 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, *Bacteroides thetaiotaomicron* DSM 2079^T, *Bifidobacterium 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 faeces [54], and clone CL16 (98.4% similarity with an uncultured bacterium from human faeces [99], and 96.7% similarity with *Faecalibacterium prausnitzii*^{T}). The reference marker for *Bifidobacteria* specific DGGE analysis consisted of *Enterococcus faecium* DSM 20477^T, *E.coli* IMBH 252/07, *Bifidobacterium 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 *Bacteroides thetaiotaomicron* DSM 2079^T, faecal clones Bt 17 and Bt11 and *Enterococcus 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 *Faecalibacterium prausnitzii*^{T}, 92.1% and 92.9% similarity with *Eubacterium desmolans*^{T} and 95.9% similarity with *Subdoligranulum variabile*^{T}.

3.2.6 TaqMan RTQ-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 labelled 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 3min and 45 cycles of 95°/60°C for 15/ 45 sec. The amplification program for clostridium cluster IV was: denaturation at 95° for 5 min and 45 cycles at 95°/ 55°C for 30/ 45 sec.

DNA of *Bacteroides thetaiotaomicron*^{T} and *Bifidobacterium longum*^{T}, 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 [100]. 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 (% 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.

Table 1: Primers and probes used for the quantification of faecal 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		150	[100]

		CCG TTC GCC -(BHQ-1)			
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	[101]
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	[102]
Clostridium cluster IV	sg-Clep-F	GCA CAA GCA GTG GAG T	239	400	
	sg-Clep-R	CTT CCT CCG TTT TGT CAA		400	[102]
	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 to 1107.

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	GTGCTGCAGAGAGTTTGATCCT GGCTCAG	57	[103]
	985r	GTAAGGTTCTTCGCGTT	57	[104]
	341f-GC	CCT ACG GGA GGC AGC AG	55	[63]
	518r	ATT ACC GCG GCT GCT GG	55	[91]
Bacteroides	32f	AACGCTAGCTACAGGCTT	56	[97]
	708r	CAATCGGAGTTCTTCGTG	56	[97]
bifidobacteria	g-BifidF	CTCCTGGAAACGGGTGG	58	[105]
	g-BifidR	GGTGTTCTTCCCGATATCTACA	58	[105]
Clostridia cluster IV	sg-Clep-F-GC	see table 1	55	
	sg-Clep-R			

3.2.7 Statistical analysis.

Food frequency data were analyzed based on Chi-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 $1-\sum ((p_i)^2)$, 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.

3.3 Results

3.3.1 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 (Chi^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).

3.3.2 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 faeces, that is 20 copies of 16S rRNA gene per reaction.

The elderly harbored only 69%±21.6% of the total bacterial load in their faeces compared to control (figure 1). *Bacteroides* were found to represent a larger percentage than *Bifidobacteria* in all samples (figure 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 (figure 1) in their faecal microbiota than young volunteers (p=0.036).

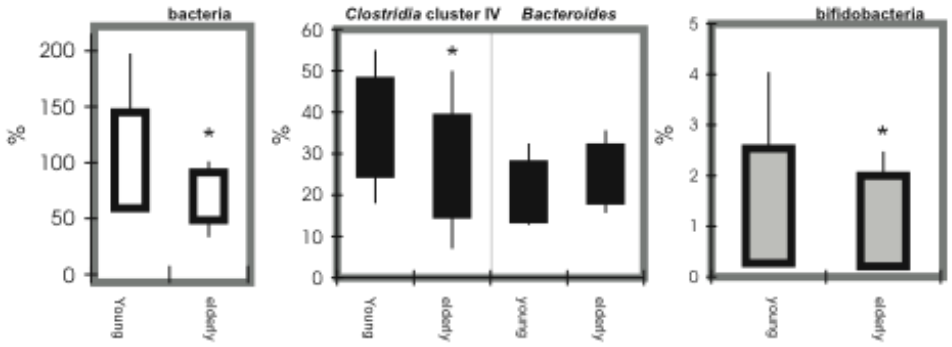

Figure 1: Proportions of *Bacteroides,* bifidobacteria and *Clostridium* cluster IV of total bacteria in stool samples of institutionalized elderly and young healthy volonteers.Total amount of bacteria is depicted relative to the mean counts for healthy young volunteers.

3.3.3 DGGE bandpattern analysis

DGGE fingerprinting with primer pair 341GC-518, which amplified the total microbial community, showed high inter-individual variations (figure 2). The mean numbers of

bands per individual were 16.6 ± 3 bands for institutionalized elderly and 20 ± 3 bands in control. Two bands as highlighted in figure 2 occurred significantly less frequently in elderly probands. Shannon and Simpson indices of diversity were significantly lower for elderly than control (figure 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 (figure 4) along the first two principal components. Cluster analysis could separate the fingerprints of young and elderly (figure 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.

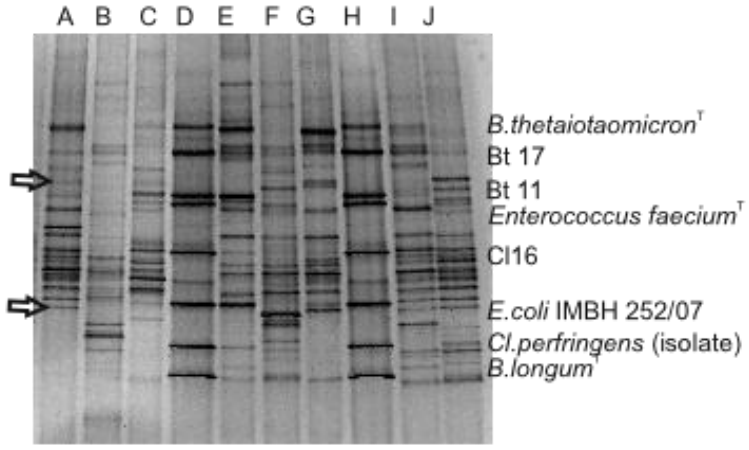

Figure 2: PCR-DGGE bandpattern of 16S rRNA genes of dominant bacteria amplified with primer pair 341GC-518. Bands that were observed more frequently in young than in elderly are indicated with arrows. Organisms and sequences listed were used for the construction of the reference lanes.

A, G, I, J, young; B, C, E, F, elderly; D, H, reference lanes

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3.3.4 Bifidobacteria

An average of thirteen bands was 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 (figure 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.

3.3.5 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 (figure 3) showed a tendency to be lower for elderly than for control. PCA results suggest a tendency for less *Bacteroides* diversity with ageing (figure 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.

3.3.6 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 (figure 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 *Faecalibacterium 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 *Subdoligranulum variabile*^{T}, and 97.9%/ 98% similarity to uncultured bacterium DQ793301).

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

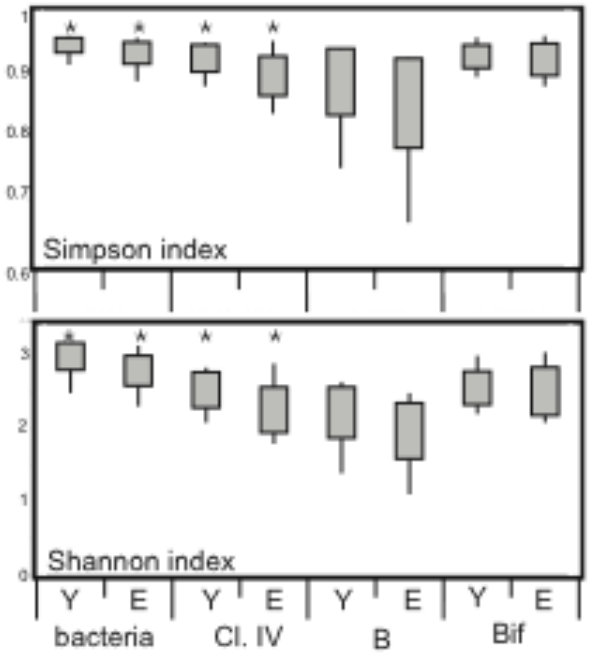


Figure 3: Diversity indices derived from DGGE fingerprinting of 16S rRNA coding regions.

Y, young; E, elderly; Cl.IV, *Clostridium* cluster IV; B, *Bacteroides*, Bif, bifidobacteria;

*, $p < 0.05$.

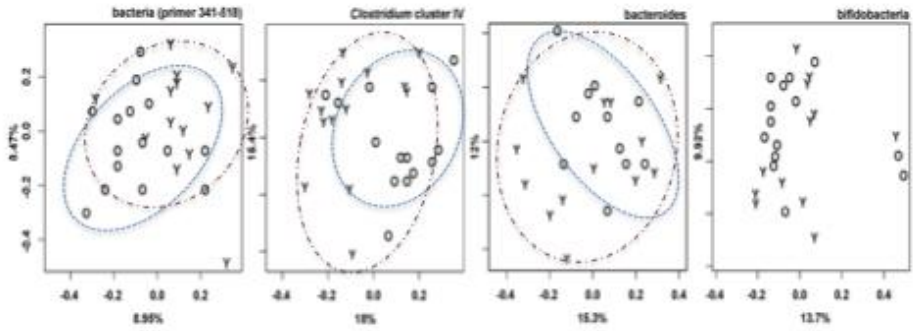


Figure 4: PCA of DGGE fingerprints of 16S rRNA genes of dominant bacteria in faecal samples.

Y, young; O, elderly

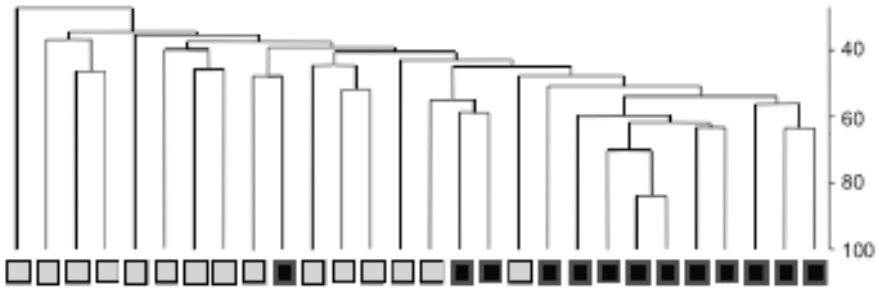


Figure 5: UPMGA dendrogram showing clusterin (Dice) based on the similarities of DGGE fingerprints of dominant bacteria obtained with primer pair 341GC-518.

□ , elderly; ■ , young

3.4 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 [94]. 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 analysed, 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 aging

[13, 106] or hospitalization of aged citizens [95]. 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. [107] recently reported that supplementation with lactitol and a probiotic *Lactobacillus* led to an increase in *Bifidobacteria* in the microbiota of elderly.

Bartosch et al. [95] also reported a marked reduction in the abundance of *Faecalibacterium prausnitzii*, a member of the *Clostridium* cluster IV which decreased in our aged group. However several studies [106, 13, 95] 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 [108]. Discrepancies in changes of the *Bacteroides* abundance might be due to country-specific differences in this bacterial subgroup as indicated by Mueller et al. [109]. Mueller et al. [109] 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* [13].

Reduced numbers of *Bacteria* in the faecal content of elderly reflect the physiological alterations associated with ageing. These include prolonged colonic transit time and reduced dietary energy requirement and food uptake [110]. 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.

3.5 conclusion

Studies comparing elderly and young volunteer microbiota with PCR-DGGE fingerprinting and qPCR 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.

3.6 Acknowledgements

I cordially thank the participants of the study, my collegues at the Department of Nutritional Sciences, especially Mag. Kathrin Liszt, Dr. Viviana Klose and Mag. Verity-Ann Sattler of the IFA Tulln and Mag. Ewald Denner of the Institute for Bacteriology and Hygiene of the Veterinärmedizinische Universität Wien for their kind support. The Hochschuljubiläumsfond of the Austrian National Bank funded this study.

4 Characterization of *Bacteria*, *Clostridia*, *Bacteroides* in faeces of vegetarians using qPCR and PCR-DGGE fingerprinting

4.1 introduction

The highly diverse microbiota of the human gastrointestinal tract has been associated with the pathogenesis of colorectal cancer [111], inflammatory bowel diseases [112, 113], obesity [27], metabolic syndrome [8] and atopic diseases [114]. Two of the most predominant subpopulations in the human faecal microbiota are the *Clostridium leptum* subgroup and *Bacteroides* [6, 115, 54]. Members of these populations contain butyrate and propionate producing fibrolytic bacteria [116, 117]. The metabolic activities of these organisms have a significant influence on colonic health as butyrate is known as the major energy source for colonocytes [118] and is involved in epigenetic regulation of gene expression in colonic epithelium [119, 23]. Furthermore it has been shown that vegetarian diet influences gene expression through epigenetic modification [120, 121]. Another important subgroup of the human microbiota is that of the bifidobacteria. Stimulation of these bacteria has previously been shown after prebiotic intervention with inulin and fructo-oligosaccharides [92]. Bifidobacteria have been shown to be involved in prevention of atopic disease [122], and prevention of obesity and insulin resistance *via* enhanced barrier function of the gut epithelium [9].

The prevalence of a vegetarian diet (e.g., little or no animal protein, low fat and high fiber content) increased substantially during the last few decades in the Western world and this trend is likely to continue in the future [123]. 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 [123]. Different lifestyle factors and diets were shown to have a significant impact on the faecal microbiota [124, 109, 125, 126]. To our knowledge only one investigation [127] of vegetarian microbiota has been performed with molecular methods. This analysis of a single individual revealed that *Clostridium* cluster XIVa, *Clostridium* cluster IV,

Clostridium cluster XVIII were the major components of the vegetarian gut microbiota [127].

In the present study, we applied qPCR and PCR-DGGE fingerprinting to investigate the dominant microbiota in 29 young omnivores and vegetarians assessing *Clostridium leptum* subgroup, *Bacteroides* and bifidobacteria.

4.2 Material and methods

4.2.1 Participants

Twenty-nine healthy young individuals, 15 vegetarians (aged 19-34 years, BMI 22.06 $\pm$ 3.82) and 14 omnivores (aged 21-31 years, BMI 21.02 $\pm$ 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, pre- and probiotics three months prior to sampling. All trial subjects agreed to participate in the study and gave their informed consent.

4.2.2 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 manufacturers' protocol with minor modifications [128, 102] and immediately stored at -20°C.

4.2.3 TaqMan qPCR

Bacterial 16S rRNA was quantified by TaqMan qPCR using primers and probes previously published (table 1). TaqMan Probe (Clept-P) for *Clostridium* cluster IV was designed with CLC DNA Workbench (http://www.clcbio.com). DNA of *Bacteroides thetaiotaomicron*^{T} and *Bifidobacterium longum*^{T}, 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 using Rotor-Gene 3000 calculation software (Corbett operator manual) and Excel.

4.2.4 PCR, DGGE fingerprinting

16S rRNA coding regions were amplified using a ready-to-use mastermix (Promega) in a Robocycler (Stratagene). Group specific primers (table 2) were applied at the temperatures indicated in table 2. 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 [98] with a linear gradient of 25-65% for bacteria, 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.

4.2.5 Statistical analysis.

Quantitative PCA results were analyzed in Excel using F-test and Student's T-test. Food frequency data and interesting bands of DGGE fingerprints were analyzed based on Chi-square approximation as implemented in SPSS15. PCR-DGGE band comparison tables were created in GelComparII (www.applied-maths.com) and analyzed with principal component analysis (PCA) using the default settings in 'R-software environment for statistical computing' (www.r-project.org) until 100% variance explained. Transformed data were plot in a bi-plot as a function of the first two principal components. Shannon 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. All tests were double sided, $p<0.05$ was considered as significant.

4.3 Results

4.3.1 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 (Chi^2 Test; $p<0.027$) consumption of vegetables than vegetarians. Three of the vegetarians assessed followed vegan diet; all others followed a lacto-ovo vegetarian diet. Five vegetarians eat fish a few times a year.

4.3.2 Relative quantitation with qPCR

Bifidobacteria, members of *Clostridium* cluster IV and *Bacteroides* were quantified as percentage of the total bacterial DNA (figure 1). 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 counts of bacterial DNA than omnivores but these differences were not significant due to high interindividual deviations. The mean proportion of *Clostridium leptum* subgroup 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 bifidobacteria in vegetarians (1.52 ± 1.29 %) was unchanged to omnivores (1.59 ± 1.73 %). The 3 vegans did not show any differences in the group of vegetarians, even more they cluster nicely at the mean values.

4.3.3 PCR-DGGE quantitative analysis

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 also in the dominant bacteria dataset some grouping was visible (figure 2). 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. *Bacteroides* bandpatterns were composed of 9.5 ± 2.9 bands, *Clostridium* cluster IV of 12.75 ± 3.37 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 (figure 3) from *Clostridium* cluster IV were more prevalent in omnivores than in vegetarians (Chi^2 Test; $p<0.005$; $p<0.022$). The first sequence was 96.7 % similar to *Faecalibacterium prausnitzii*^{T} and 99.5 % to *Faecalibacterium prausnitzii* AJ270469 and the second one matched next to *Clostridium* sp. BI-114^T (similarity 94.7%) and uncultured bacterium DQ793301 (similarity 97.9 %).

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

Target organism	Primer and probe	Sequence (5' - 3')	Size (bp)	Conc. (nM)	Reference
Bifidobacterium spp.	Forward primer	GCG TGC TTA ACA CAT GCA AGT C	125	300	
	Reverse	CAC CCG TTT CCA GGA GCT		300	

	primer	ATT			
	Probe	(FAM)- TCA CGC ATT ACT CAC CCG TTC GCC -(BHQ-1)		150	[100]
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	[101]
All bacteria	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	[102]
Clostridium cluster IV	sg-Clept-F	GCA CAA GCA GTG GAG T	239	400	
	sg-Clept-R3	CTT CCT CCG TTT TGT CAA		400	[96]
	Clept-P^{++}	(FAM)-AGG GTT GCG CTC GTT-(BHQ-1)		200	This study

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

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

Target	**Primer**	**Sequence (5'-3')**	**Ann.tem**	**Reference**

organism	pairs		p (°C)	
All bacteria	27f	GTGCTGCAGAGAGTTTGATCCTGGCTCAG	57	[103]
	985r	GTAAGGTTCTTCGCGTT	57	[104]
	341f-GC	CCT ACG GGA GGC AGC AG	55	[63]
	518r	ATT ACC GCG GCT GCT GG	55	[91]
Bacteroides	32f	AACGCTAGCTACAGGCTT	56	[97]
	708r	CAATCGGAGTTCTTCGTG	56	[97]
bifidobacteria	g-BifidF	CTCCTGGAAACGGGTGG	58	[105]
	g-BifidR	GGTGTTCTTCCCGATATCTACA	58	[105]
Clostridium cluster IV	sg-Clept-F-GC	See table 1	55	[129]
	sg-Clept-R3	See table 1		[129]

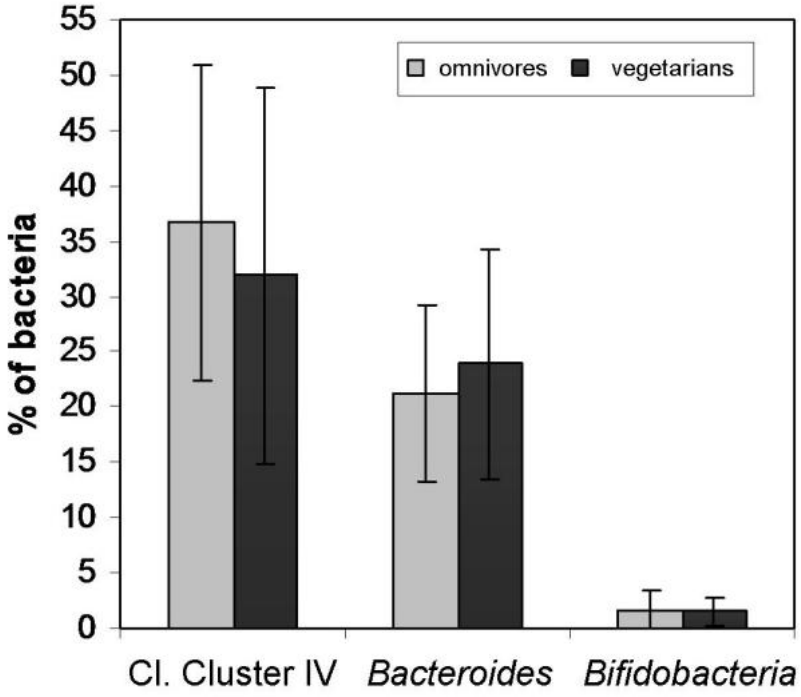


Figure 6: Proportions of *Bacteroides*, bifidobacteria and *Clostridium* cluster IV of the total bacterial load in stool samples of vegetarians and omnivores [130]**.**

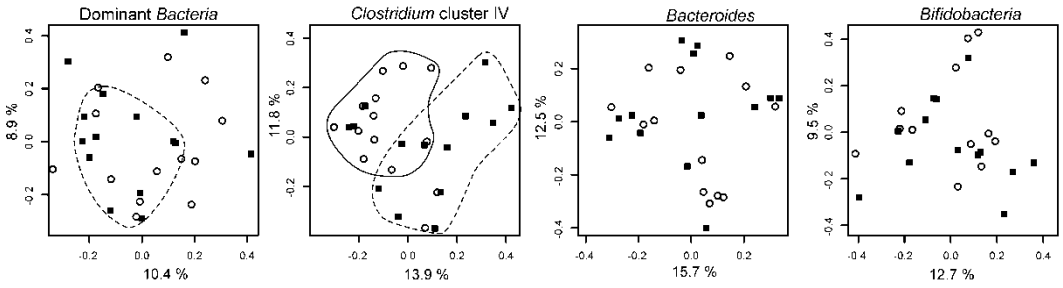


Figure 7: PCA of DGGE fingerprints of 16S rRNA coding regions of dominant bacteria, *Clostridium* cluster IV, *Bacteroides* and bifidobacteria in faecal samples.

O, omnivores; ■, vegetarians

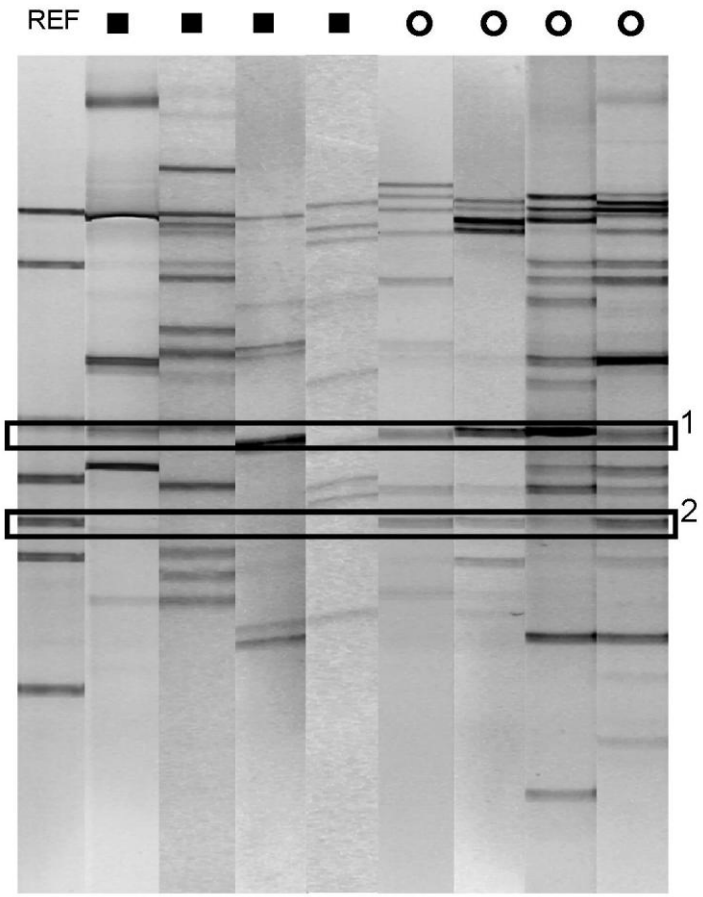


Figure 8: PCR-DGGE bandpatterns of 16S rRNA coding regions of *Clostridium* cluster IV amplified with primer pair sg-Clept-F-GC/sg-Clept-R. Bands that were observed more frequently in omnivores than in vegetarians are indicated with a black box. 1: 94.7% similarity to *Clostridium* sp. BI-114^T and 97.9 % 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 %) [130].

O, omnivores; ■, vegetarians; REF, reference lane

4.4 Discussion

Vegetarianism has been frequently associated with a decreased risk for different diseases. Therefore, we want to see how vegetarian diet influences the human intestinal microbiota. In this study we could show that vegetarian diet has an effect, especially on *Clostridium* cluster IV.

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 [131]. We could confirm with our questionnaire that vegetarians have a higher dietary fibre intake, which leads to a decreased transit time [132]. We suppose that the higher counts of bacterial DNA in vegetarians may be attributed to that fact.

Furthermore, we could see a tendency for higher proportions of *Bacteroides* and lower proportions 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 [133] [134]. Nevertheless, members of Clostridium leptum subgroup have the same ability. One explanation could be that may dietary fibre enhance the development of Bacteroides more.

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 [134] [135] [136]. 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 [109]. Therefore, we suppose that higher meat consumptions may increase the abundance of the *Clostridium leptum* subgroup in the gut microbiota. A higher prevalence of this subgroup was discussed to be associated with obesity and cancer [111] [125], where also increased diversity was observed.

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 *Ruminococcus* sp. were more prevalent in omnivores than in vegetarians. As this two subspecies are known as dietary fibre degraders we did not expect this finding. Two possible explanations for this observation may be the before discussed fact of a higher meat consumption or the very complex network of cross-feeding species. However, certainly further investigations have to be done to clarify this aspect.

Despite of enormous interindividual variations a tendency for smaller proportions of *Clostridium* cluster IV in vegetarians was observed. This might suggest a smaller capacity for energy harvest from food in vegetarians. Higher proportions 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. Summarizing, it remains to be determined if these shifts result in differential metabolite profiles that might in turn affect host metabolism and disease risks.

4.5 Acknowledgements

I cordially thank the participants of the study, my collegues at the Department of Nutritional Sciences, especially Mag. Kathrin Liszt; Dr.Viviana Klose and Mag. Verity-Ann Sattler of the IFA Tulln and Mag. Ewald Denner of the Institute for Bacteriology and Hygiene of the Veterinärmedizinische Universität Wien for their kind support.

5 microbiota analysis in immunocompromised patients under chemotherapeutic intervention

5.1 introduction

To prevent the invasion of endogenous bacteria from oral cavity and the gastrointestinal tract, three defense mechanisms are considered to be relevant: innate immunity, mechanical mucosal barrier, and colonization resistance [137]. However, chemotherapy and the use of antibiotics damage the rapidly generated mucosal cells of the gastrointestinal tract and disrupt the ecological balance, allowing pathogens such as *Clostridium difficile* to grow [138, 139]. This bacterium is thought to be the causative agent in up to 20% of antibiotic-associated diarrhea (AAD) cases [140]. It is evident that the intestinal microbial ecosystem has an important but incompletely defined role in mucosal protection [141].

Mucositis is a major oncological problem, caused by the cytotoxic effects of cancer chemotherapy and radiotherapy. Approximately 40% of patients receiving standard dose chemotherapy and up to 100% of patients receiving high dose chemotherapy and stem cell or bone marrow transplantation suffer from abdominal pain, ulceration, bloating and vomiting [142-145]. Although gastrointestinal disturbances (mucositis, diarrhoea and constipation) and immunosuppression are well recognised side-effects of cancer treatment, very little research has been conducted into the underlying mechanisms and the changes in the composition of the microbiota. Because of these changes, nutrient absorption and other intestinal functions involving the microbiota may also be altered [146].

For this reason, we investigated shifts in faecal microbiota of patients receiving cancer chemotherapy with or without antibiotics in comparison to healthy control individuals. We sampled at four time points before or after chemotherapy to study changes in faecal microbiota over the course of time. In this study we aimed to clarify how chemotherapy agents influence faecal bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV*, *Clostridium* cluster *XIVa* and *C. difficile* using culture-

independent methods assessing abundance and diversity. Furthermore, we hypothesize that the human faecal microbiota is resilient in its ability to return to its original composition after cycles of chemotherapy.

5.2 material and methods

5.2.1 Study participants and study design

Eleven subjects receiving chemotherapy with or without antimicrobial therapy (aged 55 ± 14 years, BMI 28 ± 7) from the Sozialmedizinisches Zentrum Ost in Vienna and nine healthy individuals (aged 55 ± 21 years, BMI 24 ± 5) joined this study. Faecal samples of each ambulant oncology patient were collected at four time points within two weeks before and after the onset of treatment. Two out of eleven patients had never received any chemotherapy before, while the others had a history of chemotherapy. One subject receiving chemotherapy additionally suffered from rheumatism while another subject suffered from diabetes mellitus type II, hypertension and obesity. Anonymous medical records reported types of malignancies as well as chemotherapeutic and antimicrobial treatment. Four individuals suffered from a form of leukaemia, three patients suffered from a form of lymphoma (non-hodgekin). Other malignancies were breast cancer, bladder cancer ovarian arrhenoblastoma and multiple myeloma. Among the chemotherapeutic regimens were bendamustin, bortezomib, cytarabin, dexamethosane, doxorubicin, etoposid, gemcitabine, idarubicin and melphalan. Leukaemia patients furthermore received G-CSF (neupogen) and/or radiated erythrocyte concentrate.

Stool samples of healthy individuals were collected once. Study populations were gender balanced, with 55% females in both oncology patients and healthy controls.

We interviewed all study participants assessing: age; gender; body length; weight; health status (chronic and acute diseases); life-style aspects such as cigarette and alcohol consumption as well as physical activity. Dietary habits were assessed using a

food frequency questionnaire. Exclusion criteria for healthy controls were (a) antimicrobial medication (b) chemotherapeutic treatment and (c) pre- and probiotics at least three months before sample collection. All subjects gave written informed consent. The ethics committee of Vienna approved the study.

5.2.2 Stool sample processing

After collection, stool samples were brought to the laboratory and immediately stored at -70°C. A 200 mg aliquot of each sample was treated twice for 45 s in a bead-beater (Mini-Beadbeater-8). Thereafter DNA was extracted using the QIAamp® DNA Stool Mini Kit (QIAGEN) following the manufacturer's protocol. Finally we stored the DNA at -20°C until analysis.

5.2.3 Type strains

We used type strains, known to be part of the human gastrointestinal microbiota and cloned sequences, to design a DGGE standard lane marker. Type strains *Bacteroides thetaiotaomicron* DSM 2079^T, *Enterococcus faecium* DSM 20477^T, *Lactobaccilus reuteri* ATCC 55730^T, *Bifidobacterium longum* ssp. *longum* DSM 20097^T, *Escherichia coli* IMBH 252/07 and clones *CL16* and *CC34* (see below) were used creating a comparable standard lane marker for DGGE gels analyzing all bacteria (figure 1).

5.2.4 Clone library

To create a standard lane marker for DGGE analysis and to identify dominant members of the *Clostridium* cluster *XIVa* we constructed a clone library from stool samples. For this purpose PCR products amplified with primers 195-F [147] and Ccocc-R [96,105] were inserted into a p-GEM Easy Vector (Promega) following the instructions of the manufacturer. Nucleotide sequences were corrected for primer and vector sequences in CodonCodeAligner (www.codoncode.com) and taxonomically

identified using the online tools of the ribosomal database project (http://rdp.cme.msu.edu/). The clone library used for creating a standard lane marker for DGGE analysis of *Clostridium* cluster IV has previously been described [126].

5.2.5 Polymerase chain reaction (PCR)

PCR was carried out amplifying 16S rRNA gene sequences from bacteria in faecal samples, type strains and cloned sequences for DGGE analysis as well as for creation of the clone library using group-specific and kingdom-specific primers (table 1). The PCR reaction mixture consisted of ready-to-use mastermix (Promega) with 1.5 mM $MgCl_2$, 500 nM of primers and 2 µl of template DNA. When amplifying faecal samples, bovine serum albumin (Fermentas) was added to a final concentration of 400µg/ml. We used a Robocycler (Stratagene) for all amplifications.

5.2.6 PCR-DGGE-fingerprinting

DGGE was performed as described by Muyzer et. al. [63]. Primer pairs and annealing temperatures to analyze the diversity of (a) bacteria, (b) *Clostridium* cluster *IV* and (c) *Clostridium* cluster *XIVa* are described in table 1.

PCR products were separated by polyacrylamid gels with a denaturing gradient of 30-60% for predominant bacteria, 30-50% for *Clostridium* cluster *IV* and 35-50% for *Clostridium* cluster *XIVa.* Electrophoresis was performed for 9 h at 129 V at 60°C (predominant bacteria), 5 h at 200V at 60°C (*Clostridium* cluster *IV*) and 7 h at 200 V at 60°C (*Clostridium* cluster *XIVa*). We created a standard lane marker for each DGGE analysis assay to ensure reliable gel-to-gel comparison. These standard lane markers (described above) were loaded in triplicate on each gel to adjust for gradient-variations within gels.

Table1: 16S rRNA gene primers used for PCR-DGGE fingerprinting.

Target organism	Primer pairs	Sequence (5'-3')	Ann. temp (°C)	Reference
All bacteria	27f	GTGCTGCAGAGAGTTTGATCCTGGCTCAG	57	[103]
	985r	GTAAGGTTCTTCGCGTT	57	[104]
	341f-GC	CCT ACG GGA GGC AGC AG	55	[63]
	518r	ATT ACC GCG GCT GCT GG	55	[91]
Bacteroides	32f	AACGCTAGCTACAGGCTT	56	[97]
	708r	CAATCGGAGTTCTTCGTG	56	[97]
bifidobacteria	g-BifidF	CTCCTGGAAACGGGTGG	58	[105]
	g-BifidR	GGTGTTCTTCCCGATATCTACA	58	[105]
Clostridium cluster IV	sg-Clept-F-GC	GCA CAA GCA GTG GAG T	55	[129]
	sg-Clept-R3	CTT CCT CCG TTT TGT CAA		[129]
Clostridium cluster XIVa	CcoccF-GC	AAATGACGGTACCTGACTAA	55	[129]

	CcoccR CTTTGAGTTTCATTCTTGCGAA	[129]
GC-clamp	CGCCCGGGGCGCGCCCCGGGCGGC CCGGGGGCACCGGGGG	[63]

5.2.7 Quantitative TaqMan-PCR

The abundance of bacteria and bacterial subgroups was measured by 16S rRNA gene-targeting TaqMan qPCR. Optimal annealing temperature of the PCR primer pairs and expected product sizes (table 2) were ascertained using a Robocycler (Stratagene), before the TaqMan-assay was performed in a Rotorgene 3000 (Corbett Life Science). We analyzed each sample in duplicate. Amplification reactions were carried out in a total volume of 10µl consisting of 5µl Taq-Man SensiMix DNA Kit (Quantance), 1µl of each primer and Taq-Man-probe (concentrations see table 2) and 10ng of bacterial DNA. Amplification programs included an initial denaturation at 95°C for 10 min followed by 40 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C (*bacteria*, *Clostridium* cluster *IV*), 56°C (*Clostridium* cluster *XIVa*), 58°C (*C. difficile*) or 60 °C (*bacteroides*, Bifidobacteria) for 30 s and extension at 72°C for 50 s.

We used tenfold serial DNA dilutions of type strains *Bacteroides thetaiotaomicron*^{T}, *Bifidobacterium longum ssp. longum*^{T} and *C. difficile* as well as the clones *CL16* and *CC34* and one faecal sample to construct standard curves for comparison of PCR reaction efficiencies among different experiments.

We quantified DNA of *Bacteroides thetaiotaomicron*^{T}, *Bifidobacterium longum ssp. longum*^{T} and *C. difficile*, using the nanodrop method and calculated DNA copiel/µl through mean G+C content of each strain. Quantification of clones *CL16* (*Clostridium leptum* 16) and *CC34* (*Clostridium coccoides* 34) was done determining the numbers of CFU (colony forming units). Relative percentages of bacterial subgroups were

calculated in relation to total rRNA gene copies amplified with primer pair BAC-338-F and BAC-805-R [102].

We reviewed sensitivity of PCR reactions with stepwise dilutions of standard curve DNA until we achieved sensitive detection levels of PCR. The specificity was confirmed using non-target DNA.

Table 2: Primers and probes used for TaqMan qPCR quantification of 16S rRNA genes.

Target organism	Primer and probe	Sequence (5' - 3')	Size (bp)	Conc. (nM)	Reference
Bifidobacterium spp.	Forward primer	GCG TGC TTA ACA CAT GCA AGT C	125	300	
	Reverse primer	CAC CCG TTT CCA GGA GCT ATT		300	
	Probe	(FAM)- TCA CGC ATT ACT CAC CCG TTC GCC -(BHQ-1)		150	[100]
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	[101]
All bacteria	BAC-338-F	ACT CCT ACG GGA GGC	468	1000	

		AG			
	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	[102]
Clostridium cluster IV	sg-Clept-F	GCA CAA GCA GTG GAG T	239	400	
	sg-Clept-R3	CTT CCT CCG TTT TGT CAA		400	[96]
	Clept-P^{++}	(FAM)-AGG GTT GCG CTC GTT-(BHQ-1)		200	This study
Clostridium cluster XIVa	195F	GCA GTG GGG AAT ATT GCA		500	www.microbial-ecology.net/probebase
	CcoccR	CTT TGA GTT TCA TTC TTG CGA A		500	[96]
	CcoccP	(6-FAM)-AAATGACGGTACCTGAC TAA-(BHQ-1)		150	[96]
Clostridium diffficile	CdiffF	TTG AGC GAT TTA CTT CGG TAA AGA		1000	[100]
	CdiffR	TGT ACT GGC TCA CCT	151	1000	

		TTG ATA TTC A	
	CdiffP	(6-FAM)-CCA CGC GTT ACT CAC CCG TCC G-(BHQ-1)	200

5.2.8 Statistical analysis

Statistical evaluation of differences between groups (chemotherapy and control) and changes within the chemotherapy group (all time points before and after chemotherapy) was carried out using the OriginPro version 8 (OriginLab, Northampton, MA). For two group comparisons of independent ordinal and interval values we used the two-sample-T-test and the nonparametric Mann-Whitney-U-test. For the analysis of related data we used the paired-sample-T-test or the non-parametric Wilcoxon signed-rank test. P values <0.05 were considered statistically significant.

We analyzed PCR-DGGE fingerprints in GelComparII (www.applied-maths.com) and applied principal component analysis (PCA) using the default settings in 'R-software environment for statistical computing' (www.r-project.org) until 100% variance was explained. Shannon and Simpson diversity indices were calculated on quantitative band information 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 index, given as 1 - D, this is $1 - \sum((p_i)^2)$, p_i is the relative frequency of the *i*-th species. In short, the higher the Simpson (0-1) and Shannon indices are, the higher the diversity.

5.3 Results

5.3.1 Dietary aspects

We assessed the participants` dietary habits using a food frequency questionnaire. All study participants (patients and controls) were omnivores and showed similar consumption patterns of liquids, alcohol, fruits, vegetables, grains and milk products. Healthy controls stated more frequent consumption of fruits, whole grain products and alcohol several times a week compared to patients receiving chemotherapy.

5.3.2 Chemotherapeutic treatment with or without antibiotics decreases absolute bacterial numbers in comparison to healthy controls

We investigated absolute numbers and relative percentages of bacterial subgroups to investigate whether chemotherapy *with or without* antibiotics changes the human GI microbiota composition in contrast to healthy individuals and over a time period. The ambulant patients receiving chemotherapy harboured only 25 ± 22% of the absolute bacterial load in their faeces compared to healthy controls and with it less *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV* and *XIVa*. Despite high inter- and intraindividual variations the differences in absolute numbers of bacteria ($p = 0.02$), *Bacteriodes* ($p=0.01$), bifidobacteria ($p=0.001$) and *Clostridium* cluster *XIVa* ($p=0.001$) were statistically significant. Furthermore, oncology patients had less absolute numbers of *Clostridium* cluster *IV*, but not significantly. Abundance of all bacterial subgroups declined after chemotherapeutical intervention often followed by a rebound of bacterial abundance (figure 2).

Subject ON007 shows a sharp decline at time point 4 of all bacteria and bacterial subgroups following blood stem cell transplantation and medical intervention.

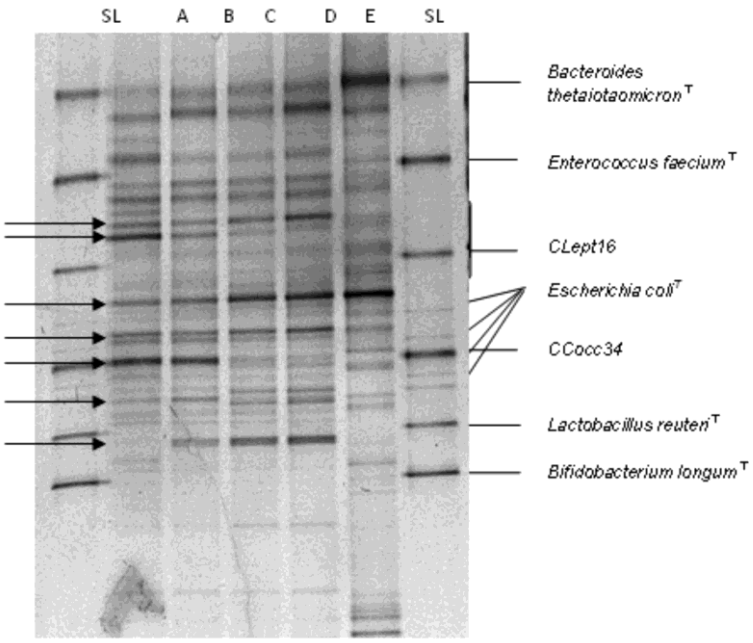


Figure 9: Changes of PCR-DGGE fingerprinting of 16S rRNA coding regions of dominant bacteria after chemotherapeutic treatment. Bands that become stronger or nearly disappear are indicated with arrows.

A, B, C and D, samples of ON001 after chemotherapy; E, healthy control; SL, standard lane.

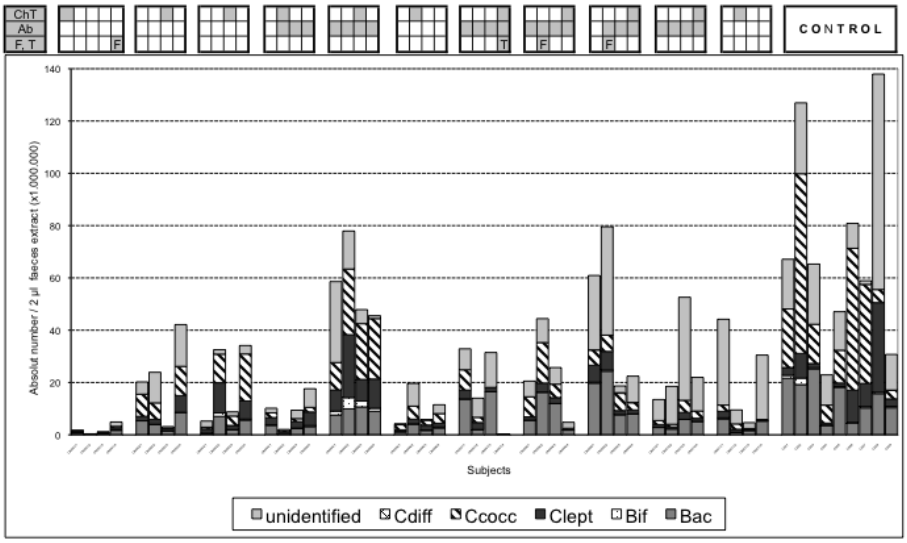


Figure 10: Impact of medical treatment (chemotherapy and antibiotics) on human faecal microbiota. Absolute numbers of *Bacteroides* (Bac), bifidobacteria (Bif), *Clostridium* cluster *IV* (Clept) and *XIVa* (Ccocc), *C. difficile* (Cdiff) and unidentified bacteria analyzed by TaqMan-qPCR [148].

ChT, Chemotherapy; Ab, Antibiotics; F, fever; T, blood stem cell transplantation; Subject ON001 received chemotherapy before first sampling. Subjects C001- C009 represent the healthy age- and gender matched control group.

5.3.3 Clostridium cluster IV and XIVa show great alterations due to chemotherapeutical interventions, while the geni Bacteroides and bifidobacteria seem to be marginally affected

In contrast to absolute numbers in figure 2, figure 3 shows the relative quantification of *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV*, *Clostridium* cluster *XIVa* and *C. difficile* as percentage of total bacterial DNA. The mean proportion of *Bacteroides* in stool samples was 27 ± 12% in chemotherapy patients and 23 ± 12% in healthy individuals. The mean percentage of bifidobacteria in patients was 1.17 ± 1% and 1% ± 0.7% in controls. Patients harboured on average 16 ± 11% of *Clostridium* cluster *IV* and

20 ± 14% of *Clostridium* cluster *XIVa*, while controls harboured 10 ± 7% and 34 ± 22% of clostridial clusters *IV* and *XIVa*.

It can be seen in figure 4A that there is a difference between the oncology and control group with regard to the subgroups *Clostridium* cluster *IV* and *XIVa*. Oncology patients harboured significantly more (p = 0.02) *Clostridium* cluster *IV* and less *Clostridium* cluster *XIVa* (p= 0,057) than healthy controls.

5.3.4 Clostridium cluster IV higher before chemotherapy than after.

Figure 4B illustrates the differences of the faecal microbiota composition before and after chemotherapeutic cycles. The mean percentage of *Clostridium* cluster *IV* before chemotherapy was 22 ± 10% compared to after chemotherapeutic cycles with 14 ± 9% of all analyzed bacteria. Hence, there is a significant difference (p = 0.01). The average amount of *Bacteroides*, bifidobacteria and *Clostridium* cluster *XIVa* were 30 ± 12%, 1.2 ± 1.7% and 22 ± 11% at time points before chemotherapy and 28 ± 12%, 1.6 ±1.5% and 24 ± 17% after chemotherapy.

5.3.5 C. difficile colonization found in individuals receiving chemotherapeutic and antibiotic treatment

To find out whether the chemotherapeutic and antibiotic disruption favours the growth of pathogens, we investigated the abundance of *C. difficile*. Two out of eleven patients (18%) receiving chemotherapy harboured *C. difficile.* Patient ON009 harboured *C. difficile* at four time points investigated with a mean proportion of 0.4 ± 0.7%, showing the highest level (1.22%) at sampling point 3 (highlighted in figure 3), when chemotherapeutic and antibiotic treatment took place. Pathogenic *C. difficile* grew up at time point 3 in patient ON011 (3.90% of all analyzed bacteria, highlighted in figure 3) after chemotherapeutic intervention at time point 2.

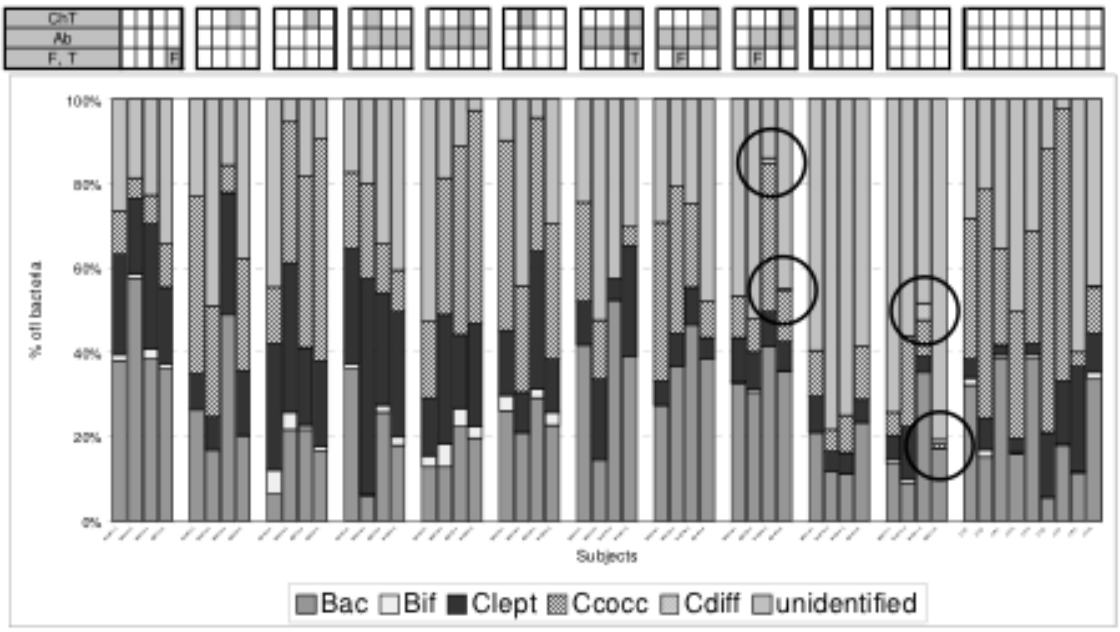


Figure 11: Percentage of *Bacteroides* (Bac), bifidobacteria (Bif), *Clostridium* cluster *IV* (Clept) and *XIVa* (Ccocc), *C. difficile* (Cdiff) and unidentified bacteria of analyzed bacteria quantified by TaqMan-qPCR. Red circle indicates *C. difficile.*

5.3.6 PCR-DGGE fingerprinting analysis shows decreased diversity of bacteria and Clostridium cluster XIVa in response to medical treatment compared to healthy individuals

DGGE fingerprinting analyses of bacteria, *Clostridium* cluster *IV* and *Clostridium* cluster *XIVa* indicate a highly diverse dataset between individuals and uniqueness of faecal microbiota. The mean numbers of bands per patient receiving chemotherapy were 21.8 ± 5 for bacteria, 9 ± 5 bands for *clostridium* cluster *IV* and of 14.9 ± 7 bands for *clostridium* cluster *XIVa*. DGGE profiles of healthy controls had 23.1 ± 5 bands for bacteria, 9 ± 5 for *clostridium* cluster *IV* and 18.9 ± 7 for *clostridium* cluster *XIVa*. The number of bands as well as Shannon and Simpson diversity indices (figure 5) show a

lower diversity of bacteria (figures 5A, 5B) and *Clostridium* cluster *XIVa* (figure 5E, 5F) in oncology patients. In line with increased abundance of cluster *IV* oncology patients had greater diversity of *Clostridium* cluster *IV* according to DGGE fingerprints (figure 5C, 5D). The dataset was subjected to principal component analysis (PCA). PCA analysis extracts underlying components of samples according to their variance. Graphs A and B in figure 6 display PCA of *Clostridium* cluster *IV* fingerprints. Graph A shows an increase in variation in oncology patients compared to healthy controls. Graph B more precisely specifies that the microbiota differs before and after chemotherapy according to their *Clostridium* cluster *IV* DGGE fingerprints. PCA analysis shows increased variability in the DGGE fingerprints of individuals under therapy compared to healthy controls. Chemotherapy resulted in distinctive clustering of bandpatterns before and after chemotherapy.

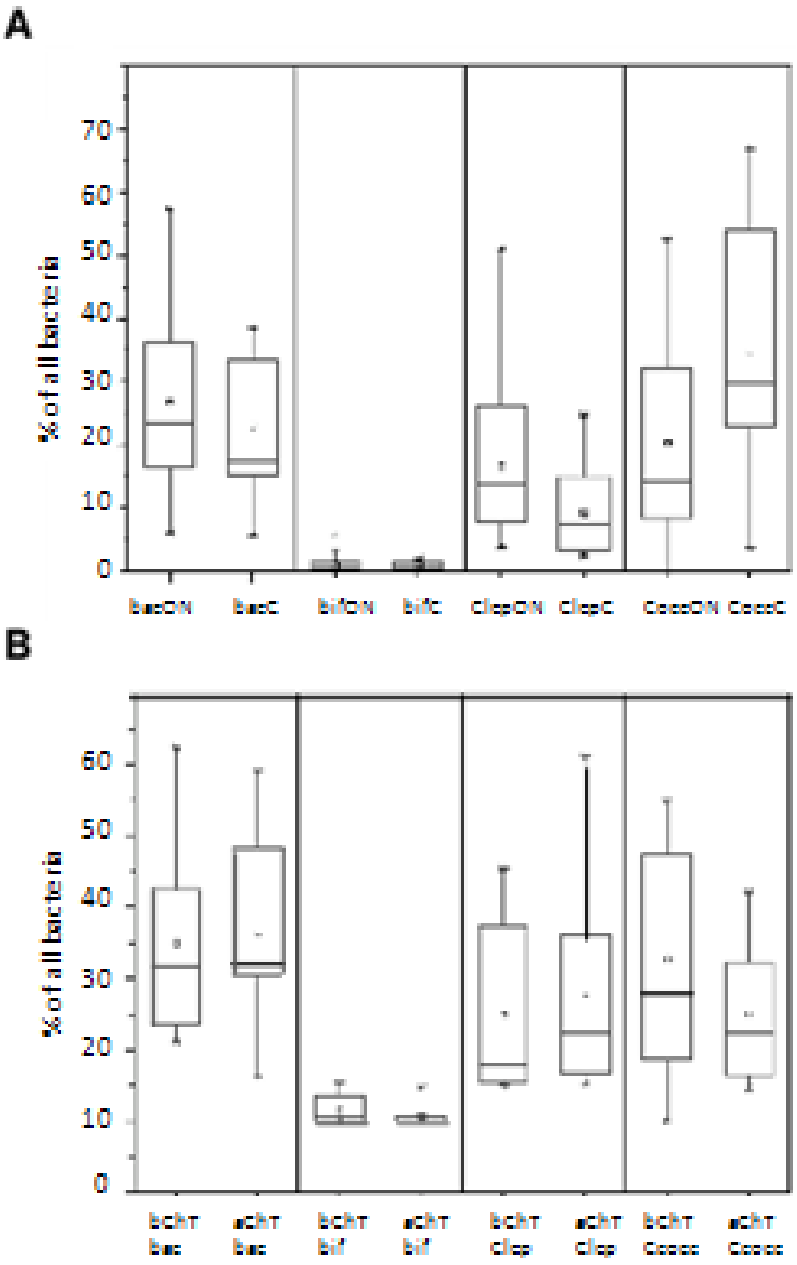


Figure 12: (A) Relative abundances of faecal bacteria in patients receiving chemotherapy ± antibiotics in comparison to healthy individuals. (B) Comparison of samples before (bChT) and after chemotherapeutic cycles (aChT). Relative abundances of *Bacteroides* (bac), bifidobacteria (bif), C*lostridium* cluster *IV* (Clept) and cluster *XIVa* (Ccocc) are given relative to total bacteria.

ON, oncology patient; C, healthy control; bac, *Bacteroides;* bif, bifidobacteria; Clept, *Clostridium* cluster *IV*; Ccocc, *Clostridium* cluster *XIVa*

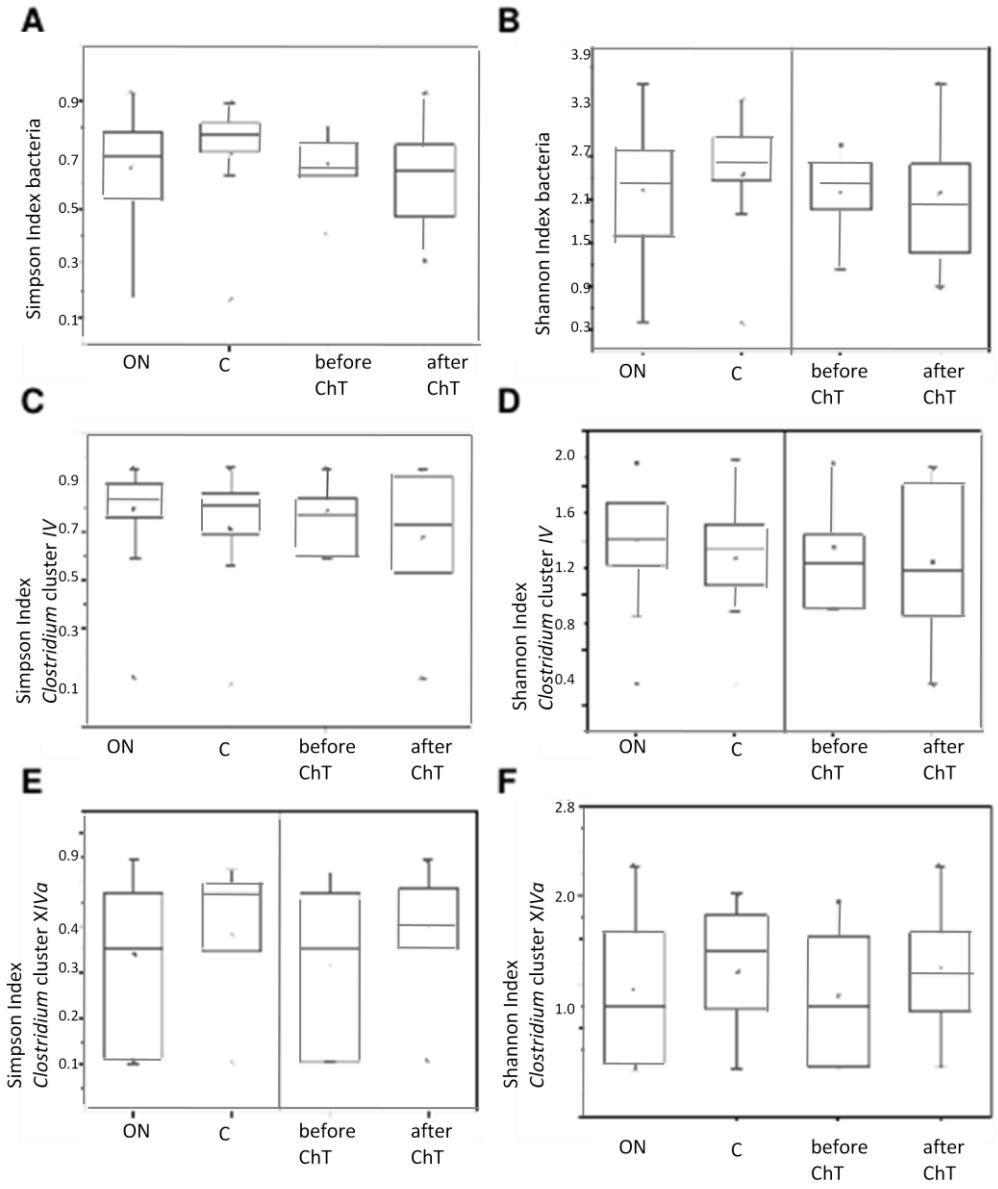


Figure 13: Simpson and Shannon diversity indices derived from PCR-DGGE bandpatterns of 16S rRNA coding regions of (A, B) bacteria, (C, D) *Clostridium* cluster *IV* and (E, F) *Clostridium* cluster *XIVa.*

ON, Oncology patients; C, healthy controls; ChT, chemotherapy

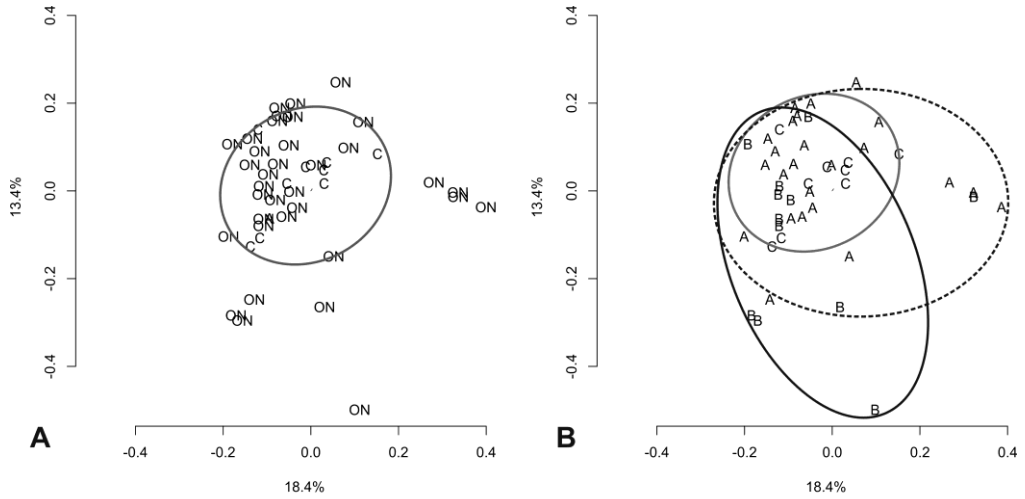


Figure 14: Principal component analysis (PCA) of *Clostridium* cluster *IV* DGGE fingerprints of 16S rRNA coding regions in faecal samples of ambulant oncology patient and healthy controls.

O, oncology pateints; C, healthy control; A, after chemotherapy; B, before chemotherapy.

5.4 discussion

Chemotherapeutic and antibiotic use has both benefits and risks. Even if chemotherapy and antibiotics are used as life-prolonging measures for critically ill patients and to fight life-threatening infections, both are associated with severe side effects such as mucositis, diarrhoea or constipation. These side effects increase the cost of health services, and are often life-threatening [144]. Chemotherapeutic and antibiotic treatment has a detrimental impact on the host microbial ecosystem, which is important for host mucosal protection [141] and thereby increases the risk of infection [137]. Overgrowth of species with potential pathogenicity such as toxigenic *C. difficile* and inflammatory complications are among the most common serious complications of chemotherapy and antibiotic treatment among patients with cancer [138, 137].

In this study, we investigated how the use of cancer chemotherapy mostly with antibiotic treatment perturbs the faecal microbial ecosystem during the course of therapy. We assessed if the microbiota is able to return to its original profile after chemotherapeutic and antibiotic intervention with special interest in the abundance of *C. difficile*. We used a combination of molecular methods to compare abundance (qPCR) and diversity (PCR-DGGE) of bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV*, *Clostridium* cluster *XIVa* and *C. difficile* between groups and different time points of chemotherapy.

As mentioned above, we used faeces as source of information. Faecal microbial communities are composed of autochthonous gut members and by transient bacteria. Even though the analysis of faecal samples may overestimate the actual composition of the GIT microbiota of humans we chose faecal samples to investigate the microbial composition of the intestinal microbiota because they are easy to collect, do not involve any ethical issues and reflect shifts in microbial population composition [149].

In this study we used type strains *Bacteroides thetaiotaomicron*^{T}, *Bifidobacterium longum* ssp*. longum*^{T} and *C. difficile* as well as the clones *CL16* and *CC34* for quantification of faecal microbiota. However, a mixture of different strains for qPCR standards would show a better image of the human microbiota. Therefore absolute amounts should be considered as semiquantitative.

The majority of previous studies on the effect of chemotherapy on human faecal microbiota used standard microbiological culture techniques [139, 144]. While other studies focused on the colonization with pathogenic bacteria [137, 150] among patients with cancer and chemotherapy-induced diarrhea [151, 144], to our knowledge, we are the first to show changes in faecal bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV*, *Clostridium* cluster *XIVa* populations in addition to *C. difficile* analysis. However, the results of our *Bacteroides* analysis are not in agreement with the results of Nyhlèn et al., 2007 [139] who showed increased counts of *Bacteroides* spp. of samples obtained during chemotherapy, but with the microflora stable in most patients. Discrepancies in changes of the *Bacteroides* abundance might

be due to differences in detection techniques. Nyhlèn et al. also reported significantly increased counts of yeast in patients, making it a focus for further research in immunocompromised patients.

Our results indicate significant effects of chemotherapy and antibiotic treatment on intestinal microbiota. Despite high individual variations, we show a significantly lower absolute bacterial load in faeces of patients receiving chemotherapy in comparison to healthy controls. These findings are in line with data from van Vliet et al, 2009, who reported 100-fold lower total bacterial numbers during chemotherapy treatment than in healthy controls. In addition to lower abundance of bacteria and determined bacterial subgroups, PCR-DGGE fingerprints indicate lower diversity of bacteria and *Clostridium* cluster *XIVa* in oncology patients. The abundance of faecal microbiota decreases after cycles of chemotherapy. After the end of chemotherapeutic administration the bacterial abundance recovers within a few days sometimes even showing a "rebound-effect". Relative numbers of *Clostridium* cluster *IV* and *XIV* show great alterations due to chemotherapeutical interventions, while the genera *Bacteroides* and bifidobacteria seem to be less affected. Interestingly, oncology patients harboured significantly more *Clostridium* cluster *IV* at higher diversity compared to healthy controls. This suggests that this class of bacteria is less susceptible to disruption by chemotherapeutic intervention. The incidence of *C. difficile* in subjects ON009 and ON011 is accompanied by a decrease of the genera bifidobacteria and *Clostridium* cluster *IV.* Further research is needed to elucidate if there is a causal relationship between growth of *C. difficile* and decreased abundance of bifidobacteria and *Clostridium* cluster *IV*.

Species richness was assessed using PCR-DGGE fingerprinting. Each lane of a PCR-DGGE gel represents a microbial fingerprint of a faecal sample; each band within a lane corresponds to one bacterial species, although different species may sometimes be represented by the same band [137]. It has also been observed that one bacterial strain may form several bands due to multipe 16S rRNA operons eg *Escherichia coli*^{T} (FIG. 3). The limitations of DGGE in microbial analysis have been previously described

[98]. Nevertheless, substantial information about species composition can be obtained from very complex microbial communities such as the gut microbiota [98].

The oncology patients assessed here suffered from a variety of malignancies and received different chemotherapy treatment regimes. Only two participants (ON001 and ON008) had never received any cancer therapy before, while all others had a history of chemotherapeutic treatment. Therefore we could not hypothesize changes due to the beginning of the treatment. Most cancer patients also receive antibiotics. Microbial profiles were similar in individuals under chemotherapy regardless if they received antimicrobial treatment or not. The effects on the intestinal microbiota described here are thus unlikely to be due to antibiotics alone. Van Vliet et al. [137] tested the effect of chemotherapy in vitro and showed a direct bacteriostatic effect of chemotherapeutics on bacterial growth.

Further research is needed to show whether changes in bacterial colonization play a role in the development and maintenance of mucosal barrier function, infection and inflammation.

The use of prebiotics, probiotics and bacterial products, such as butyrate to prevent mucosal barrier injury and its complications could be a promising concept in restoring impaired functions or enhancing specific desirable functions of the microbiota [126]. The use of pro- and prebiotics to affect the composition and metabolic activity of the faecal microbiota in times of cancer chemotherapy and immunosuppression might be part of future research.

In conclusion, chemotherapy treatment causes changes in faecal microbiota, which coincides with the development of *C. difficile* infection in some patients. These changes in microbiota may have systemic effects and may contribute to the development of chemotherapy-induced mucositis, influencing important beneficial functions of the microbial ecosystem.

5.5 Acknowledgements

I cordially thank the participants of the study and Elvira Kitzweger of the Sozialmedizinisches Zentrum Ost, Vienna. Furthermore I would like tothank my collegues at the Department of Nutritional Sciences, especially Mag.Cornelia Lassl; Dr.Viviana Klose and Mag.Verity-Ann Sattler of the IFA Tulln, Mag. Ewald Denner of the Institute for Bacteriology and Hygiene of the Veterinärmedizinische Universität Wien and Dr. Konrad Domig for their kind support. This study was funded by the FWF, Vienna, Austria.

6 analysis of functional genes involved in butyrate production, and SCFA quantification

6.1 introduction

The indigenous microbiota in the mucosa, lumen and faeces of the human gastrointestinal tract (GI) is extremely complex with 300 to 1000 different species per individual. The number of microbial genes in the GI tract is between 2 million and 4 million, representing an enormous metabolic potential far greater than that of its host. The GI tract is a very dense ecosystem composed of up to 10^{14} bacteria/ml. The number of viable bacteria and species diversity differs at different sites of the GI tract [152]. The community composition differs also among human subjects [6] and is influenced by diet- related behaviours [153], health status, age [92] as well as genetic factors [154].

The most frequently encountered organisms in the human GI tract belong to non-sporulating anaerobic species mainly of the genera *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium* and *Ruminococcus.* Species of this group of GI bacteria produce particularly high amounts of short-chain fatty acids (SCFAs) from the breakdown of dietary carbohydrates (quantitative primary SCFA progenitors), protein, peptide and glycoprotein precursors [155].

Human colonic butyrate producers use butyrylcoenzyme A CoA transferase [156] for the last step of butyrate formation. A novel CoA-transferase gene is described for the colonic bacterium *Roseburia sp. A2-183*, with similarity to acetyl-CoA hydrolase as well as 4-hydroxybutyrate CoA-transferase sequences.
SCFAs produced by resident bacteria include acetate, propionate and butyrate, which are biologically active compounds [155]. Cell culture studies have indicated that butyrate at physiological concentrations enhances the growth of normal enterocytes and inhibits colon cancer cells [157]. Butyrate may have an anticarcinogenic and anti-inflammatory potential, affect the intestinal barrier and play a role in satiety and oxidative stress [158, 159]. Butyrate induces apoptosis in colorectal tumour cell lines,

reduces metastasis, and protects from genotoxic carcinogens by enhancing expression of phase II detoxification enzymes [160]. Acetate and butyrate promote methane production by GI *Archaea*. The formation of propionate is considered as a competitive pathway for hydrogen use by methanogens [161]. Organic acid degradation depends on the presence of syntrophic consortia of acetogenic bacteria and methanogenic *Archaea* [162]. The predominant archeaon, up to 11,5% of total human GI microbiota, is *Methanobrevibacter smithii* [6, 163]. Because of the rather low consumption of fermentable dietary fibers in today's Central European diet, the microbial diversity in the lumen may vary, and with that the butyrate concentrations.

We recently showed differences between the microbiota diversity in vegetarians and elderly compared to those consuming a mixed (omnivore) diet [126, 164].

Based on these results we analysed human faeces specimens of young healthy individuals with omnivore or vegetarian diets as well as institutionalized elderly by HPLC-RI and TaqMan real-time PCR to determine SCFAs content, microbiota diversity and butyryl-CoA CoA-transferase gene abundance. To assess a possible correlation between butyryl CoA CoA transferase gene abundances and the presence of *Archaea* a relative quantitation of archaeal 16S rRNA gene sequences was done.

6.2 material and methods

6.2.1 Study subjects:

Group of individuals and sample material used in this study were the same as that used by Zwielehner et al. and Liszt et al. [126,164]. The geriatric group (elderly) consisted of 17 institutionalized subjects (age: 86 ± 8 yrs / BMI 21.75 $\pm$ 5.08) from a geriatric department in Vienna. The vegetarian diet group (vegetarian) consisted of 16 young healthy subjects with a vegetarian or vegan diet (age: 26 ± 5 yrs/ BMI 21.02 ± 2.71). Seventeen young healthy subjects (age: 24 ± 2.5 yrs/BMI 22.68 $\pm$ 3.4) with a Central European diet formed the mixed diet group (omnivores).

All volunteers were interviewed using a questionnaire assessing: age, gender, body height, weight, individual health status (including chronic or acute diseases and blood lipid levels), life-style aspects such as physical activity and dietary habits. Group populations were gender balanced. Only non-pregnants and probands without digestive/gastrointestinal symptoms and no antibiotic or chemotherapeutic treatment up to three months prior to sampling participated on this study. All probands agreed to participate in the study and gave their informed consent. Approval was obtained from the viennese Human Ethics committee (Vienna 3., Thomas-Klestil-Platz 8/2).

6.2.2 Sample material

Faeces was collected from each proband individually and stored at -18°C until processed. For molecular genetics samples were kept frozen. For HPLC-based analyses sample material was defrosted at room temperature.

6.2.3 Quantification of specific metabolic genes and archaeal 16S rRNA gene by real-time PCR (qPCR)

Three different degenerated primer pairs were applied to cover the CoA-transferase gene in common gut bacteria [156]. The CoACT primer pair targets butyryl-CoA CoA transferase genes of *Roseburia* sp. *A2-183, Desulfitobacterium hafniense* (ZP_00098805, ZP_00099788), *Clostridium kluyveri* (P38942), *Clostridium tetani* (NP_781174), *Archaeoglobus fulgidus* (NP_069974) and *Yersinia pestis* (NP_405485); e.g. for CTFB primer pair: *Clostridium beijerinckii* (AF157306_3), *Streptococcus pyogenes* (NP_268527, NP_269686), *Streptomyces coelicolor* (T35020), *Streptomyces* sp. (T47110), *E. coli* (NP_416726) and *Haemophilus influenzae* (NP_438932). The PCT primer pair targets butyryl-CoA CoA transferase genes of *Clostridium perfringens* (NP_561012), *C. tetani* (NP_781170, NP_781374), *Bradyrhizobium japonicum* (NP_767528), *Listeria innocua* (NP_471607) and *Fusobacterium nucleatum* (NP_603711). A ramped annealing approach was chosen for amplification of specific

metabolic genes. Primer sets are given in Table 1. Initial denaturation for 10 min at 94°C, then 35 cycles of denaturation for 30 s at 94°C, annealing with 20 s at 55°C, 5 s at 50°C, 5 s at 40°C, elongation for 1 min at 72°C, and a final extension for 5 min at 77°C. Archaeal 16S rRNA gene sequences were amplified with a TaqMan qPCR as listed in Table 1. Conditions (primer and probe concentration 4 pmol): initial denaturation for 8 min at 95°C, 35 cycles of denaturation for 20 s at 95°C, annealing at 20 s at 57°C and extension for 20 s at 72°C, a final elongation for 1 min at 72°C. The different primer sets were applied to stool DNA samples. Quantification of PCR products was performed spectrophotometrically by using the Nanodrop method (NanoDrop Technologies, Inc.). PCR products served as standards for absolute and relative quantitation in qPCR analyses. Archaea were quantified using a PCR product from one of the samples was as a standard.

Table 1: Primers and probes used for quantification
of CoA-transferase gene sequences and 16S rRNA genes of Archea

Target organism/gene	**Primer /Probe**	**Sequence (5`-3`)**	**Reference**
CoA Transferase based on**:** *Roseburia* sp. A2**-**183	CoATD for	AAGGATCTCGGIRTICAYWSIGARATG	[156]
	CoATD rev	GAGGTCGTCICKRAAITYIGGRTGNGC	
CoA Transferase based on**:** *C. acetobutylicum*	CTFB for	GTAAACTTIGGIRTIGGIYTNCCNAC	[156]
	CTFB rev	AACAGTAACATCIAYRTGICCNCCNC	
CoA Transferase based on**:** *C. perfringens*	PCT for	GTAGGATTARRIACITWYRTIGAYCC	[156]

	PCT rev	TCCACCACCATCRTARSARTCRAAYTG	
Archaea	ARC787F	ATTAG ATACC CSBGT AGTCC	[102]
	ARC915R	AGGAA TTGGC GGGGG AGCAC	

6.2.4 Determination of SCFAs by HPLC

Concentration of SCFAs in faecal specimens were measured according to the method of Huel-Mel Chen et al.1998. Briefly, 3 g defrosted faeces was homogenised for 2 minutes in 20 ml of 0.15 mmol l^{-1} H_2SO_4 in bidest-water using a Stomacher Lab-Blender 400 (Carl Roth GmbH, Germany). The homogenate was centrifuged at 6000 x g at 2 °C for 60 min. The supernatant fluid was then filtered twice through a micro filter syringe with 0.22 µm. The filtrate received no further treatment and was directly injected into the HPLC.

The HPLC apparatus (Dionex 3000) consisted of a mobile phase reservoir; a Dionex ICS 3000 sp pump; an automatic sample injector, Dionex AS; a MetaCarb 67H organic acid chromatographic column, 300 mm; a temperature control module; a refractive index detector; a chromatography control station and a system interface module. The mobile phase was a 0.005 mMol solution of “ultra pure” sulphuric acid (VWR, Austria) in Milli-Q purified water (pH 3.1, background conductance <100 MS), filtered through a 0.2 µm (Herba Chemosan, Austria) membrane and degassed before use. Separation of SCFAs was best at a flow rate of 0.8 ml/min and a column temperature of 36 °C. To regenerate the column, the column temperature was increased to 65 °C while washing with 25 mmol l^{-1} ultra pure sulphuric acid at a flow rate of 0.8 ml min^{-1} for 2 h. Calibration of the system based upon the average of five measurements of each reference compound (Table 2).

6.2.5 Data analysis and statistics

Statistical analyses including mean, standard deviation and coefficient of variation (F-test) was applied to the data using the OriginPro8 data analysis and graphing

software. Results for each group were normally distributed (Kolmogorov-Smirnov test). A two sample t-test was performed with OriginPro8. Correlations were calculated using Pearson's linear regressions model and Spearman's rank (OriginPro8). Data analysis of Ct values and comparative correlation results were calculated using Rotor-Gene 3000^{TM} real-time analysis software system (Corbett Research, Australia) and transferred to Microsoft Excel. Food frequency data collected with a food frequency questionnaire was analysed using the Chi-square approximation as implemented in the Statistical Package for the Social Sciences SPSS release 15.0 (SPSS Inc., USA). Feaces samples were analysed in duplicates, $p>0.05$ was considered as significant.

6.3 results

6.3.1 Dietary analysis

Exercise levels (vegetarians to omnivores) were comparable. Analysis of the participant's dietary habits indicated similar consumer behavior in the respect of fruit and milk products in the individual groups. Omnivores stated significantly less frequent (Chi^2 Test; $p<0.027$) consumption of vegetables than vegetarians. Three of the vegetarian probands assessed followed a vegan diet; all others followed a lacto-ovo vegetarian diet. The omnivore group stated significantly less frequent (Chi^2 Test; $p<0.04$) consumption of meat than elderly probands and regular consumption of whole grain products several times a week. The elderly of this study did not consume any whole grain products at all but received supplements with soluble fibre (Benfiber®, Novartis).

6.3.2 Detection of butyryl-CoA CoA-transferase in faeces by RT-PCR

Applying the CoATD primer set (Figure 1), vegetarians had $7.46 \times 10^8 \pm 2.29 \times 10^8$ copies$^{-\mu l}$; omnivores $5{,}26 \times 10^8 \pm 2{,}38 \times 10^8$ copies$^{-\mu l}$ and elderly $5.26 \times 10^8 \pm 2.38 \times 10^8$ copies^{-} of the butyryl-CoA CoA-transferase. Quantification with a CTFB primer yielded

a gene abundance of 4.12 x 10^8 ± 1.81 x 10^8 copies$^{-\mu l}$ in omnivores, 4.6 x 10^8 ± 2.51 x 10^8 copies$^{-\mu l}$ in vegetarians and 3.7 x 10^8 ± 1.89 x 10^8 copies$^{-\mu l}$ in elderly. Applying the PCT primer pair, abundances of 8.61 x 10^8 ± 3.12 x 10^8 copies$^{-\mu l}$ in omnivores, 8.53 x 10^8 ± 5.28 x 10^8 copies$^{-\mu l}$ in vegetarians and 6.55 x 10^8 ± 4.73 x 10^8 copies$^{-\mu l}$ were found in elderly (figure 3).

6.3.3 Content of SCFAs in faeces as determined by HPLC

Faecal concentrations of butyrate in specimens ranged from below detection limit up to 0.094 µmol g^{-1} faeces (wt/w). The average value for omnivores was 0,023 µmol g^{-1} ± 0,027 µmol g^{-1} faeces, for elderly 0,007 µmol g^{-1} ± 0,011 µmol g^{-1} faeces and for vegetarians 0,008 µmol g^{-1} ± 0,008 µmol g^{-1} faeces. Butyric acid concentrations found in the faeces of omnivores were highest among all individuals. In spite of high inter-individual differences these values were significantly different from butyrate in faeces of elderly ($p = 0.04$).

6.3.4 Relation of specimen specific Archaea

Analysis of 16S rRNA genes showed that Archaea were nine times more abundant in Omnivores than in Vegetarians. Elderly harboured 2.8 times more Archaea than Vegetarians. These differences were not significant due to high inter-individual variance.

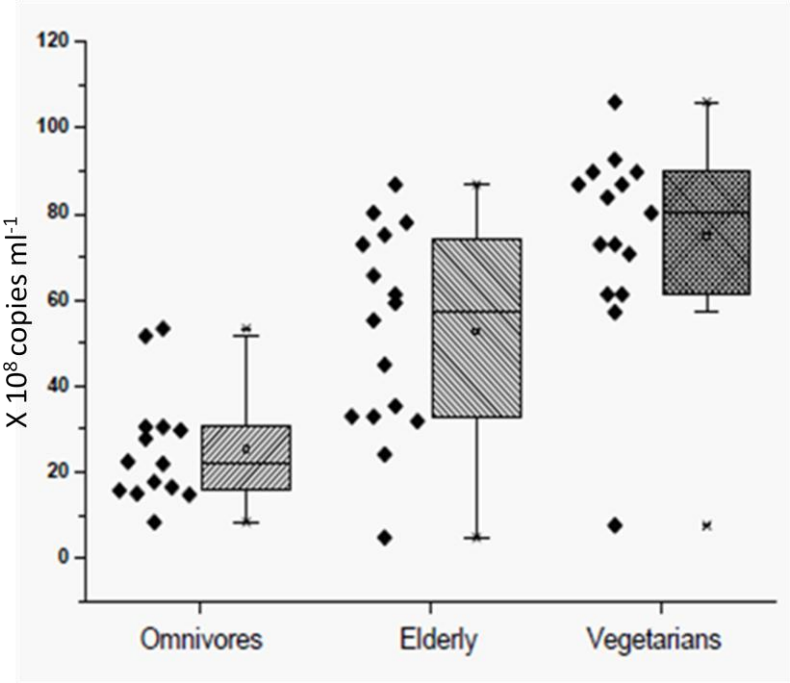


Figure 15: Butyrate-CoA CoA transferase gene abundance as assessed with the CoATD primer pair. The boxplot shows median ± standard deviation [218].

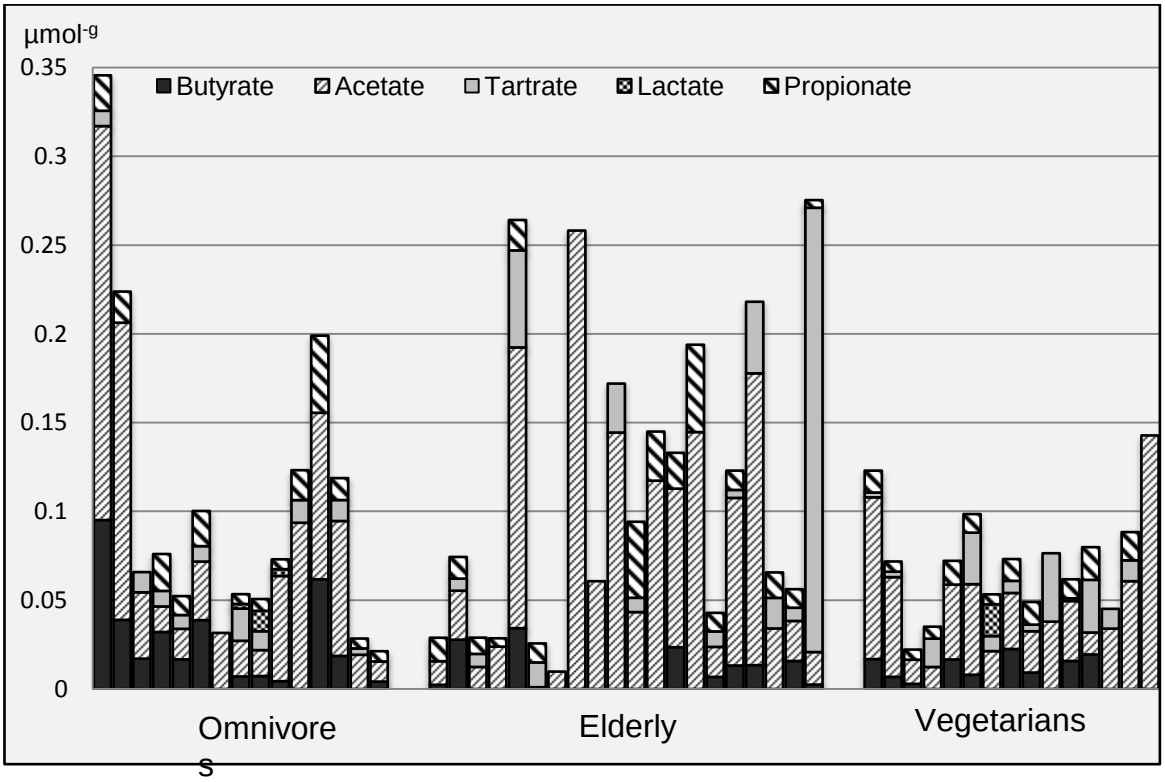


Figure 16: Short chain fatty acid concentrations measured with HPLC [218].

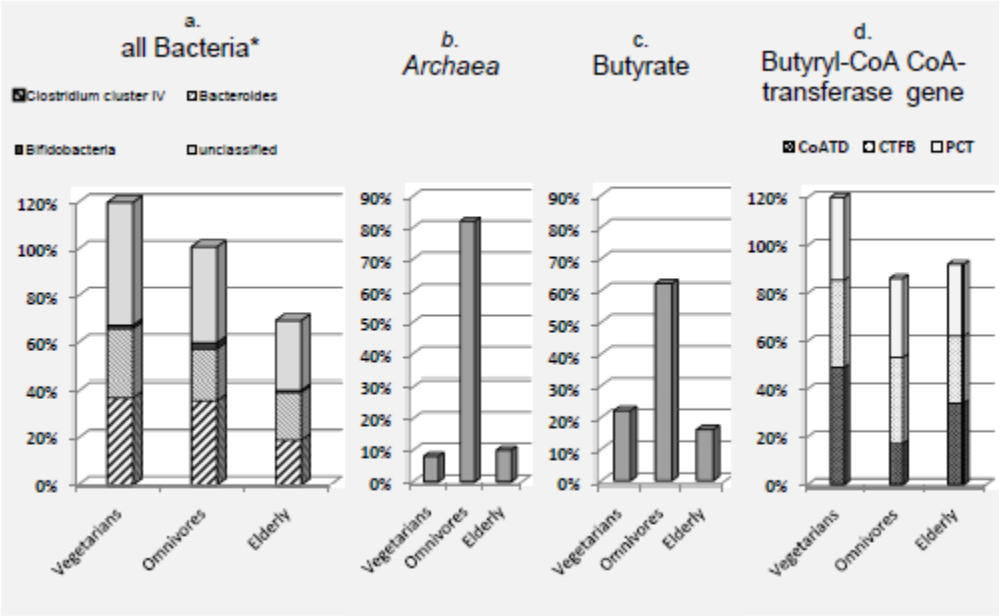


Figure 17: concentration trends in 100% total of each measurement of specific bacterial relation, *Archeae* concentration, Butyric Acid Concentration and butyrl-CoA CoA-transferase gene abundance for Vegetarians, Omnivores and Elderly [218].

6.4 discussion

Production and consumption of SCFAs is associated with GI microbiota diversity that is influenced by diet, age, activity and other lifestyle as well as host genetic factors. It was previously reported [126,164] that vegetarians have 12% more and elderly 31% less bacterial DNA in their faeces than omnivores, as shown in Figure 3. *Clostridium* cluster IV was not significantly different for vegetarians compared to omnivores. Elderly had significantly less *Clostridium* cluster IV compared to both other groups. *Bacteroides* abundance was significant higher in vegetarians.

To study the effects of microbiota changes on metabolites, such as SCFAs, we compared SCFAs in faeces using HPLC-RI, and quantified Butyryl-CoA CoA transferase genes using degenerated primer pairs in real-time PCR. *Archaea* were quantified as potential consumers of fermentation products.

6.4.1 CoA-transferase genes, SCFAs and *Archaea* in tested specimen groups

The major differences observed between the measured groups (omnivores, elderly and vegetarians) were the low level of butyrate in faeces of vegetarians compared to omnivores as seen in Figure 3c. However, the CoATD primer set amplification of butyrate genes was highest in vegetarians (Figure 3d). This unexpected finding might be attributable to a higher abundance of *Bacteroides,* one of the main SCFAs producers in the human gut, and a higher abundance of bacteria in vegetarians [164] (figure 3a) assuming that the bacterial surplus in those individuals carries genes that are amplified by this primer pair. Elderly had low levels of butyrate and low butyrl-CoA CoA-transferase gene abundance, but showed the highest levels of acetate among all subjects. Zwielehner et al. [126] reported the microbiota of elderly to be less diverse and less densely populated. Apparently the loss of diversity in the microbiota of elderly markedly affected the SCFA production, in spite of high functional redundancy in the human GI microbiome [165].

Furthermore previous work carried out by our group showed that vegetarian's species composition of the *Clostridium* cluster IV differed from the one found in omnivores [164]. Adult omnivores are reported to consume 103 g meat day^{-1}, 60 g more than recommended. Elderly Austrians are reported to consume 78 g meat day^{-1}. The overall protein uptake in vegetarians matches the D-A-CH recommendations of 0.8 g kg^{-1} body weight [166]. In contrast to protein, carbohydrates are fermented completely and thus lead to a high SCFAs abundance and high amounts of hydrogen and carbon dioxide, which are reduced by *Archaea* [52]. Up to 10% of all anaerobes in the colon in up to 85% of healthy adults are *Archaea* [167, 168]. The incidence of *Archaea* was highest in omnivores (Figure 3b). Similar to findings by Armougom et al. [167] we also found high concentrations of propionate in persons with high number of *Archaea*. Low levels of propionate in vegetarians might lead to low availability of hydrogen and thus may lead to a reduction of the archaeal gut population. In faeces of vegetarians the lowest archaeal 16S rRNA gene and the highest butyryl-CoA CoA transferase gene abundances were observed. Elderly were in between omnivores and vegetarians. The targeted genes might not be equally expressed in all individuals and the butyrate

could be removed from the gut lumen by the gut mucosa and microorganisms. Furthermore not all bacteria might use the targeted gene butyryl-CoA CoA transferase in the final steps of butyrate production and the three primer pairs might not cover all butyrate producing bacteria [156]. There was an enormous variability among elderly individuals with their data points being distributed within the ranges of young vegetarians and omnivores. One explanation might be a highly variable dietary behaviour throughout their lives, in some persons being predominantly vegetarian and in others truly omnivore. All subjects in the group "elderly" received prebiotic supplementation.

6.4.2 4.2. Methodological Aspects

qPCR for CoA-transferase genes

Targeting metabolic genes rather than 'molecular clock' genes such as the 16S rRNA gene is a growing research need but we still lack whole genome sequences of bacteria. Furthermore, not all bacteria use the same gene locus for the same metabolic task. In spite of these limitations degenerate primers for the CoA-transferase gene of *Roseburia* sp. A2-183 were designed by Charrier et al. [156]. The authors also analyzed the specificity of primers for different strains, as summarized in methods. As degenerate primers are mixtures of similar, but not identical, primers the specificity of the PCR amplification can be reduced. To avoid unspecific binding a ramped annealing approach was chosen. The specificity of the primer pairs was tested in qPCR by comparing the melt curve peaks to a negative control (*Bacteroides tethaiotaomicron* DSM 2079) and a positive control standard (specimen PCR product, including all identical target butyryl-CoA CoA transferase gene regions). However, the three primer pairs used do not cover all butyrate production genes in exist, as described in Charrier et al. [156] because not all bacterial butyryl CoA transferase gene loci are known.

HPLC analysis of SCFAs in faeces

The RI detector and the organic acid chromatographic column have regularly been used to measure SCFAs. Limited specimen volumes, their dilution and preparation influence detection limits. Weaver et al. [169] describe butyrate concentrations in human faeces ranging from 11 to 25 mM and molar ratios of acetate to propionate to butyrate of between 48:29:23 and 70:15:15, with mean values of approximately 60:20:20 [169,170]. We found mean SCFA ratios in omnivores to be 63:13:24 and in elderly to be 78:14:8. Vegetarians differed considerably from these values with molar ratios of 72:15:13. However, the *in situ* production of total colonic SCFAs is difficult to determine because more than 95% of the SCFAs are rapidly absorbed and metabolized by the host [171]. As a consequence, faecal concentrations of SCFAs are not necessarily representative of those in the more proximal colon, and are also affected by intestinal transit time [172].

6.5 Concluding remarks

Applying molecular methods to detect specific metabolic genes with HPLC analysis of SCFAs and a food frequency and activity questionnaire, we obtained evidence that diet as well as ageing affect the human GI microbiome. How the differential SCFA metabolisms in individual microbiomes affect host mucosal health i.e. via epigenetic mechanisms remains to be determined.

6.6 Acknowledgements

We thank all the study participants for their cooperation. We thank Prof. Frank Unger and Dr. Diana Berner of Department of Pharmaceutical Technology and Biopharmaceutics for their support and guidance of the HPLC apparatus. Furthermore we would like to thank Dr. Guadalupe Pinar and Dr. Katja Sterflinger for giving us access to DNA quantification machinery and Mag. Berit Hippe for her significant input and work in conducting this study. The Hochschuljubiläumsfond of the Austrian National Bank funded this study.

7 Linking phylogeny with function- stable isotope probing for the identification of butyrate-consuming microorganisms.

7.1 Introduction

Short chain fatty acids (SCFAs) are derived from fermentation of non-digestible carbohydrates in the colon. The amount of SCFAs (primarily acetate, propionate and butyrate) produced in the colon depends on the site of fermentation, the diet and the composition of the residing microbiota, and can account for 5-15% of the total energy requirements for humans [173].

Due to rapid absorption and metabolism of SCFAs, their concentrations in the colon may differ. Acetate and propionate are metabolites for gluconeogenesis in the liver [15]. Butyrate is the major energy source for colonocytes and is known to regulate various processes in the human distal gut. Butyrate may have an effect on inflammation [158], oxidative stress [158], intestinal barrier function [159, 174, 175], visceral perception and rectal compliance [176] and may play a role in satiety [177, 178]. In vitro and animal studies showed that butyrate downregulates the expression of genes associated with proliferation and oxidative stress and upregulates the expression of Mucin associated genes (Muc 1–4), tight junction proteins (zonulin and occludin) and the butyrate transporter monocarboxylate transporter-1 (MCT1) [23].

The formation of toxic products and lower availability of SCFAs in the distal colon were suggested to be involved in the pathogenesis of ulcerative colitis and cancer [179, 180, 181]. In a recent genome-wide transcription study, oxidative stress related pathways and NFkB signalling were shown to be affected by butyrate. More specifically, the expression of glutathione peroxidases GPX 1 and GPX 3 and glutathione reductase (GSR) were upregulated in the oxidative stress pathway [23] thus offering a possible explanation of the health effects of butyrate. The pro-apoptotic effect of butyrate in colon cancer cells might be attributable to the decreased oxidation rate of butyrate in tumor cells, hence resulting in increased intra-cellular butyrate concentrations,

causing increased histone deacetylation and subsequently decreased proliferation [23]. Consequently, butyrate may have a positive anti-cancer effect in the colon.

Hu et al. [182] recently reported the hypothesis that the beneficial effect of SCFAs on the metabolic syndrome was due to higher concentrations of SCFAs in the portal vein, which activates AMP activated protein kinase (AMPK) in the liver. AMPK functions as a major cellular fuel gauge and a master regulator of metabolic homeostasis [182]. Since SCFA levels in the distal colon are crucial for positive health effects, SCFA producing species have been in the focus of research in the past. It has previously been described that the colon epithelium very efficiently removes butyrate from the faecal stream [183]. Microbial SCFA consumption rates by the human microbiota have –to our knowledge- not yet been investigated in depth.

In the environment, consumption of butyric acid has been described for river sediments and rice field mud [51], methanogenic sludges [184], and bioreactors by syntrophic consortia of acetogenic bacteria and methanogenic archaea [185-187]. *Syntrophomonas* spp., *Tepidanaerobacter* sp. and *Clostridium* sp. from cluster III and IV were suggested as butyrate consumers in those studies. *Clostridium* cluster III comprises the *Clostridium cellobioparum, C.tremitidis, C.cellulolyticum, C.papyrosolvens, C.aldrichii, C.thermocellum, C.stercorarium* and *C.thermolacticum* [188]. *Clostridium* cluster IV comprises *Clostridium leptum, C.sporosphaeroides* and *C.cellulosi* [188]. Zou et al [189] describe that apart from acetate, butyrate is the major methanogenic substrate in paddy field soils.

Consumption of butyric acid in the colon can directly be measured by quantifying its levels over time. In faecal samples there is likely to be enough dietary fibre as a substrate for SCFA production. Butyrate production rates may exceed bacterial assimilation when the continuous removal of metabolites by the epithelium is not mimicked. Knowing which bacteria are capable of consuming butyrate will enhance our understanding of butyrate consumers in this system. In addition, quantification of microbial butyrate consumers will allow estimation of net colon butyrate production. Improved knowledge on metabolic capacities of the gut microbiota is needed to

correctly interpret individual analysis of gut microbiota composition as a basis for individualized dietary counseling and symbiotic supplementation.

A direct way of linking identity of microorganisms to a specific function is stable isotope probing (SIP) of nucleic acids, and in particular of RNA [88, 190, 87]. Nucleic acid SIP capitalizes on the incorporation of heavy stable isotopes (13C, 15N, 18O) into RNA (or DNA). It is based on physical separation of isotopically labelled 'heavy' RNA from unlabelled, 'light' RNA, and the subsequent identification of actively label incorporating populations by cloning and sequencing of 'heavy' RNA.

RNA-SIP has recently been applied in an in vitro model of the large intestine to identify bacterial consumers of starch and glucose [89, 90]. Barclay et al. [191] identified SIP in their review as a powerful technique to link the metabolic activity and diversity of "unculturable" bacteria in inflammatory bowel diseases.

In this pilot study the ability of gut bacteria to consume butyric acid was assessed applying RNA-SIP in vitro in a stool sample of a healthy young donor. Cloning and sequencing of 'heavy' RNA was used to identify butyrate-consuming species.

7.2 Material and methods

7.2.1 Butyrate pulse conditions and sampling regime.

A faecal sample was donated by a healthy young volunteer and immediately brought in an anaerobic chamber. The sample was divided into 0.5 g aliquots in sterile 2ml screw-cap tubes containing 5 sterile glass beads at 37˚C in order not to disrupt the microstructure of the sample. Aliquots were pulsed with 100mmol/g ^{13}C butyrate, ^{12}C butyrate or nuclease-free water. The volume of the pulse was always 500µl. At 2, 4, 8, 12, 24 and 48h after the pulse two aliquots were removed from the anaerobic chamber. One aliquot was used for gas chromatography- flame ionization detector GC-FID analysis of SCFAs and the other one was used for RNA extraction.

7.2.2 GC-FID analysis of SCFAs

Immediately after the incubation aliquots were homogenized for 10 seconds and stored at -80˚C upon analysis. Methylvaleric acid was added as internal standard in a concentration of 5µl/ sample. Supernatants were carefully thawed on ice and beadbeat twice at maximum speed for 90s with 1.5 intervening minutes on ice. Samples were centrifuged twice at 30 000 x g for 30 min at 4˚C. Supernatants were diluted ten fold with water and formic acid to a final concentration of 1% formic acid in 300µl vials. SCFAs were separated on an Alltech AT^{TM}-1 column applying a ramped program 40 -180˚C at 20˚C /min.

7.2.3 Nucleic acid extraction, isopycnic centrifugation and fractionation

Immediately after the incubation, each aliquot was homogenized for 10s with 500µl RNAlater (Applied Biosystems) and immediately stored at -80˚C upon RNA extraction. RNA was extracted following a previously described protocol [192], quantified in a Nano-Drop Spectrophotometer (Thermo Scientific) and checked on a 1% agarose gel. RNA yield was 30-45 µg g^{-1}. Extracted RNA (500ng) was density separated by isopycnic centrifugation in cesium trifluoroacetate [87]. Gradients of density-resolved RNA were fractionated, the cesium trifluoroacetate buoyant density (BD) of each fraction determined, and RNA precipitated from fractions as described earlier [87]. The fractionated centrifugation gradients from the ^{13}C-butyrate pulse experiment covered an average density range of 1.819 g ml^{-1} (fraction 1) to 1.711 g ml^{-1} (fraction 14; figure 3).

7.2.4 Reverse trancriptase PCR (rt-PCR) and PCR-DGGE fingerprinting

Fractionated RNA was reverse transcribed using and AMV transcriptase (Fermentas) and primer 985r [104]. cDNA was amplified using 341f [63] with a GC-clamp attached

to its 5'end and 518r [91] in 100µl volumes and precipitated. PCR products were analyzed in DGGE applying a denaturing gradient of 30-62.5% for 12h at 100V.

7.2.5 $^{12}C/^{13}C$ isotope ratio mass spectrometry (IRMS) analysis

RNA samples were prepared for isotope ratio mass spectrometry (IRMS) analysis by cutting with sucrose as described by Manefield et al [87] to ensure sufficient material for IRMS.

7.2.6 Clone libraries

Clone libraries were constructed from fraction 2 of the 13C-labelled sample after four hours of incubation. Amplifications were carried out using primer pair 341 and 985r which bind to most bacteria. Amplified products were cloned into a p-GEM Easy Vector (Promega) following the instructions of the manufacturer. Clone libraries were screened as previously described [68]. Clone inserts were sequenced by 'DNA confidence' (Vienna). Nucleotide sequences were corrected for vector and primer sequences in CodonCode 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/) and FASTA nucleotide similarity search (http://www.ebi.ac.uk/Tools/fasta33/nucleotide.html). GC contents were calculated using a pearl script.

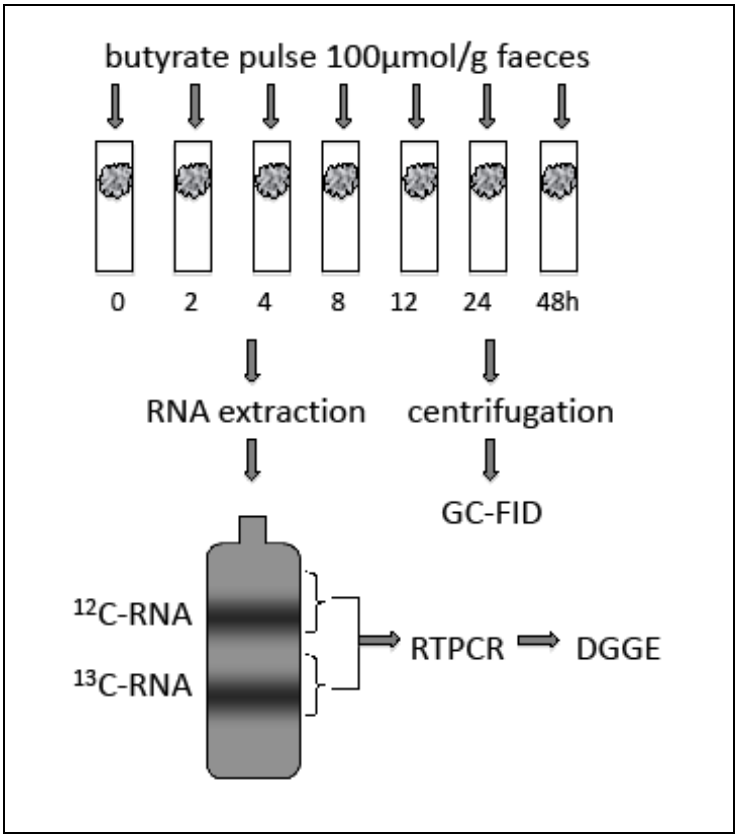


Figure 18: Schematic diagram of the experimental setup and methods.

7.3 Results

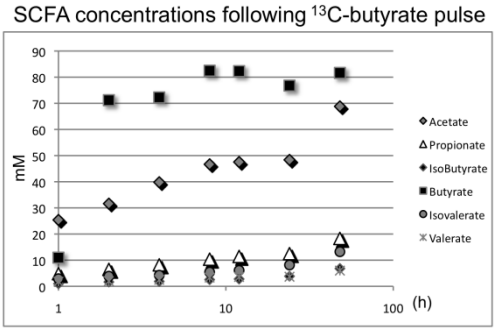
To identify butyrate consuming microorganisms of the human gastro-intestinal (GI) tract, a faecal sample was anaerobically pulsed with 100µmol/g ^{13}C-butyrate. Two controls were pulsed with either ^{12}C-butyrate or water. Destructive sampling was applied in order not to disrupt the microstructure of the sample.

7.3.1 GC-FID of short chain fatty acids (SCFAs)

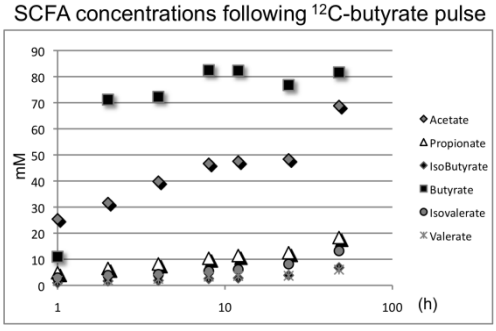
Initial concentrations of SCFAs in faecal supernatant were 25.4 mM acetate, 4.8 mM propionate, 1 mM isobutyrate, 11mM butyrate, 2.9 mM isovalerate and 1.8 mM valerate. The SCFAs concentrations continued to rise until the end of the incubation at

48h (57.4 mM acetate, 14.4 mM propionate, 5.8 mM isobutyrate, 22.2 mM butyrate, 11.1 mM isovalerate and 5.2 mM valerate; figure 1). Butyrate concentrations reached a plateau at 23 mM after 4h in the untreated control sample (figure 1c). Butyrate concentration in the ^{13}C-butyrate pulsed sample reached a plateau after 8h incubation (figure 1a). Production of SCFAs during the experiment indicates that microbes or microbial enzymes were still active *ex vivo*. The concentrations of SCFAs followed similar curves in experiment and controls (figure 1a, b, c). Thus the heavy pulse did not induce a significant disruption as compared to the control pulse to the SCFA producing metabolic pathways in the sample. This is an important prerequisite for all SIP experiments, in order not to describe a shift in microbiota composition that was induced by the experimental setting, having no representation in the actual environment studied.

19a)



19b)



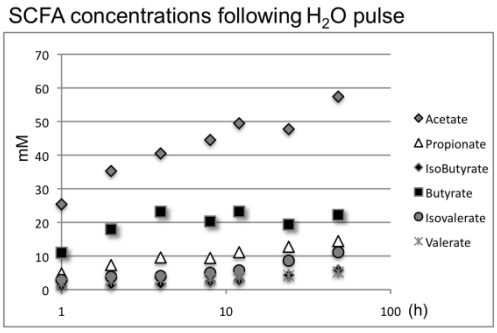
19c) 

Figure 19: SCFAs in samples and controls as assessed with GC-FID.

7.3.2 Reverse transcription PCR (RT-PCR)

Reverse transcription PCR (RT-PCR) amplification of bacterial 16S rRNA fragments showed that all fractions of all samples, including control samples, contained RNA (figure 3). Thus some RNA was contained in the entire gradient.

The intensities of individual fractions in agarose gel electrophoresis showed that RNA concentrations in the heavy butyrate pulse samples decreased with decreasing fraction densities. Highest amplicon intensities were found at densities ranging from 1.819 to 1.788 g ml^{-1}. A second peak became visible in fractions 12 (1.725 g ml^{-1}) to 14 (1.711 g ml^{-1}). This indicated that RNA of heavy label consumers accumulated at higher density whereas RNA of bacteria thriving on non-labelled substrates peaked at lower density.

In H_2O control and ^{12}C-butyrate control samples the intensity of the individual fractions increased with decreasing fraction density (figure 3). Although there has been amplifiable RNA in all fractions, RNA concentrations peaked in the lighter fractions at densities from 1.780 to 1.718 g ml^{-1}. In contrast to the samples pulsed with heavy label, RNA did not peak in the heavy fractions. This finding suggests that heavy label might have been assimilated in the samples pulsed with ^{13}C-butyrate. After 8h of incubation the amplified 16S rRNA fragment was equally intense in all fractions, showing again that gradient centrifugation and fractionation did not resolve

RNA according to its density equally well in all samples. This is a critical source of bias; therefore it was indispensible to discuss the retrieved sequences with utmost diligence to avoid false-positive results.

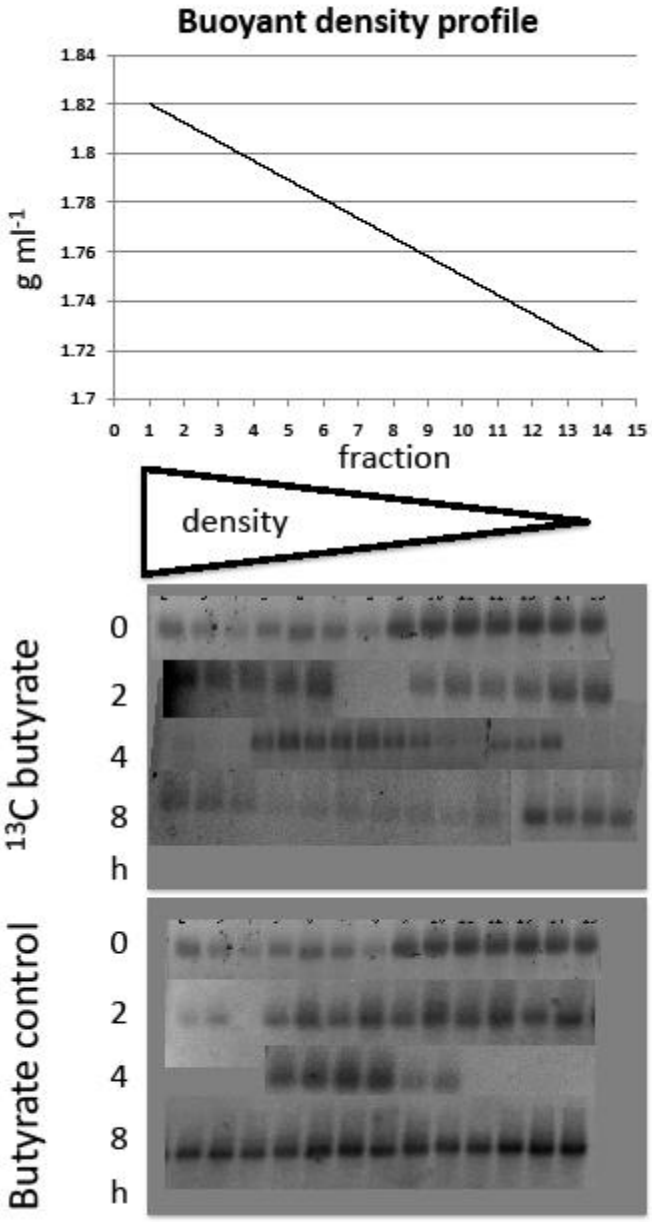


Figure 20: Buoyant density gradients and cDNA in fractions 2-15 in 2% agarose gels.

7.3.3 DGGE fingerprinting

To compare the microbial communities in the gradient fractions from all samples, PCR-DGGE fingerprint analysis was applied.

After 4 hours of incubation the bandpatterns of the heavy fractions differ from the light fractions. Sequences that might have been involved in butyrate consumption started to appear. This corroborated the incorporation of 13C-label into bacterial RNA. Control samples had uniform bandpatterns in all fractions as shown in figure 4.

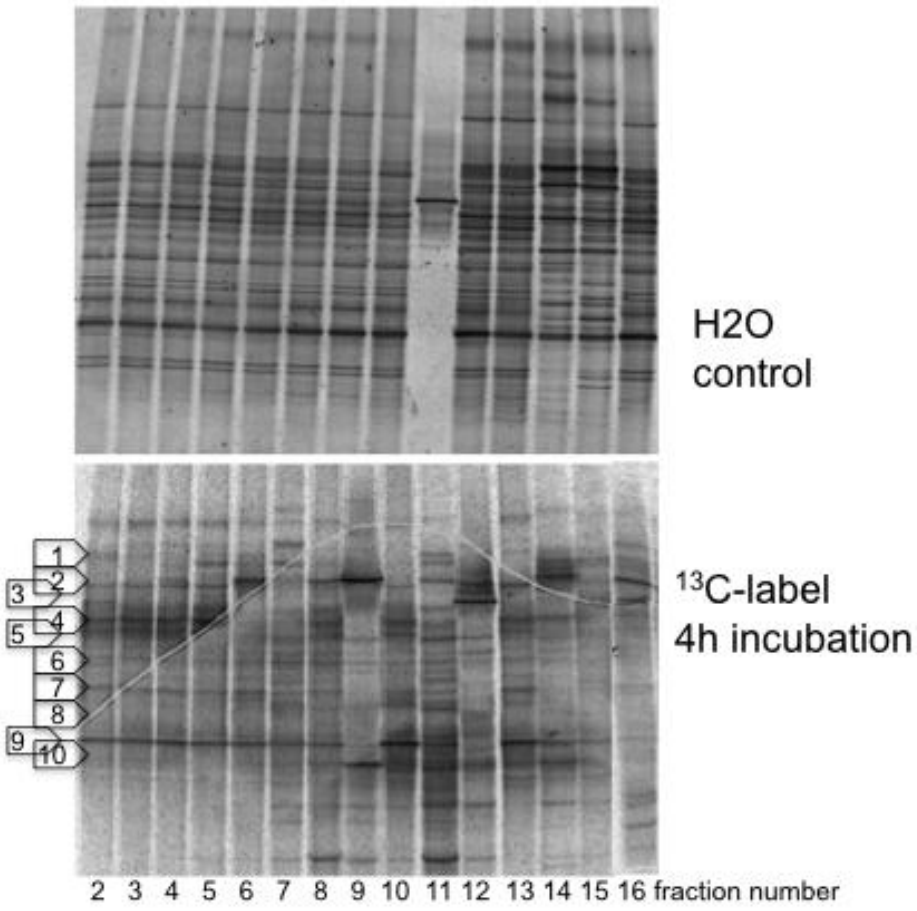


Figure 21: PCR-DGGE fingerprints obtained from fractions 2-16, four hours after incubation with ^{13}C-label. The upper image shows the control sample. Arrows and numbers indicate sequences that are enriched in the heavy fractions.

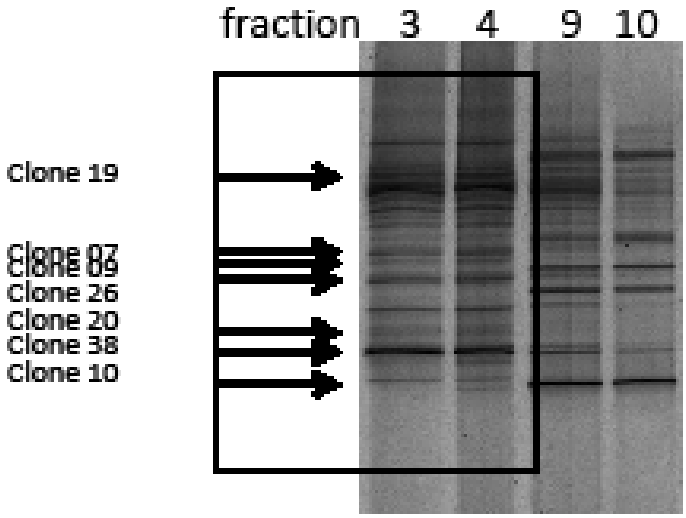


Figure 22: PCR-DGGE fingerprints from fractions 3, 4, 9 and 10, four hours after incubation with ^{13}C-label. Arrows indicate individual bands that increase in intensity in the heavy fractions 3 and 4.

The heavy fractions 3 and 4 and lighter fractions 9 and 10 of a sample after four hours of incubation were run next to each other on the same gel to allow a closer inspection of the heavy and light fractions.

Seven bands increased in intensity in the heavy fractions 3 and 4 compared to the light fractions 9 and 10. The cloned sequences corresponding to those bands are summarized in table 1.

The ^{12}C-butyrate pulsed control samples after four hours of incubation is depicted in figure 6. A closer inspection of the two heavy fractions 2 and 3 and two light fractions 12 and 13 is shown in greater detail. In this ^{12}C-butyrate control sample only three bands appeared to be enriched in the heavy fractions. These bands appeared only in

fraction number 2 and not in any of the other heavy fractions. The band indicated with the upper arrow had the same melting behaviour as clone 20.

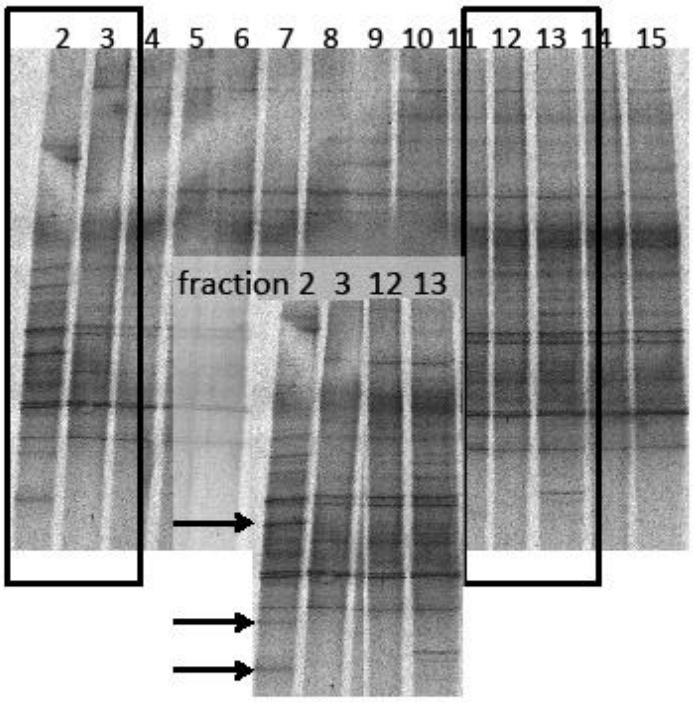


Figure 23: PCR-DGGE bandpattern of fractions 2 to 15 of the control butyrate sample after four hours incubation. Light and heavy fractions indicated with rectangles are shown in the center for better comparison.

Fraction 2 of the sample incubated for 4h with heavy butyrate was used for the construction of a clone library. Aeveral sequences were cut out in parallel and reamplified for identification. Sequences that are enriched in the heavy fractions are indicated with arrows in figure 3. Table 1 shows their similarities to previously described sequences. G+C content in this dataset ranged between 48.8 and 60% (average 54 ± 3.5%, median 53.55%). These values were compared with G+C contents in a 16S rRNA dataset (full sequences) obtained in 2005 by Eckburg et al. [54]. In the Eckburg dataset composed of 1226 full-length 16S rRNA sequences the G+C content ranged from 49.5 to 65.4% (average 52.8± 1.2%, median 53%). The clones classified as bifidobacteria had GC contents above 54%, the average + standard deviation from the

Eckburg dataset. Due to their high GC content all bifidobacteria are likely to be false-positive results. Clone 50, an unclassified *Clostridales* has GC content of 54.12% but its sequence has among all clones highest phylogenetic similarity with known syntrophic bacteria. The 16S rRNA sequence of *Tepidanaerobacter syntrophicus*^{T} for example consists of 55.7% GC.

Table: phylogenetic identification of 16S rRNA gene clone library.

Sequence	Length (bp)	Closest match	Accession number	Similarity (%)	Reference
Sequence 1 (clone 45)	679	uncultured bacterium clone, *Erysipelotrichaceae* from human faeces	AM075627	100	[193]
		Uncultured *Coprobacillus* from human faeces	FJ366449	96.7	[27]
		Uncultured *Erysipelotrichaceae* from human faeces	AY916175	96.7	[54]
Sequence 2 (cut1)	120	Uncultured *Ruminococcaceae* from gorilla gorilla	EU775497	97.5	[125]
		Uncultured *Ruminococcaceae* from human faeces	DQ801012	97.5	[194]
		Uncultured *Ruminococcaceae* form swine intestine	AF371778	97.5	[195]
Sequence 3 (clone 31)	566	Uncultured *Alistipes* sp. from human faeces	EU202015	100	[196]

		Uncultured *Alistipes* sp. from human colon biopsy	AY975278	97.0	[54]
		Alistipes putredinis^{T}	L16497	96.6	[197]
Sequence 4 (clone 49)	626	Uncultured bacterium '*Lachnospiraceae incertae sedis'* from human faeces	EU201977	100	[196]
		Uncultured bacterium from human faeces *Lachnospiraceae incerta sedis*	DQ307776	100	[198]
		Uncultured bacterium from human skin *Lachnospiraceae incerta sedis*	GQ036333	98.1	[199]
Sequence 5 (clone 5)	647	Unclassified *Sutterella* sp. from human faeces	EU202357	100	[196]
		Uncultured *Sutterella* sp. from human faeces	EU768635	96.2	[200]
		Sutterella parvirubra^{T}	AB300989	93.4	[201]
Sequence 6 (clone 27 & 8)	653	Uncultured *Sutterella* from canine duodenum	FJ719241	99.7	Suchodolski et al. unpublished
		Uncultured *Sutterella* from feline faeces	FJ031979	99.5	Ritchie et al.; unpublished
		Uncultured *Sutterella* from human faeces	FJ454360	98.8	[202]
Sequence 7 (clones 26, 50)	619	Unclassified *Clostridiales* from human faeces	EU532804	99.6	[154]

		Unclassified *Clostridiales* from human faeces	EU201582	99.4	[196]
		Unclassified *Clostridiales* bacterium from human faeces	DQ905219	97.6	[203]
Sequence 8 (clone 38)	359	Uncultured bifidobacterium from human faeces	EF063930	100.0	[204]
		Bifidobacterium catenulatum^{T}	AF432082	96.8	[205]
		Bifidobacterium pseudocatenulatum^{T}	D86187	96.8	[206]
Sequence 9 (cut 11 clone10)	632	Uncultured *Bifidobacterium* sp. from human faeces	EF063930	100	[204]
		Bifidobacterium catenulatum YIT 4016	AB437357	96.1	Watanabe et al. 2008 unpublished
		Bifidobacterium catenulatum^{T}	AF432082	95.9	[205]
Sequence 10 (clone 40)	493	*Bifidobacterium adolescentis*^{T}	AP009256	99.0	Suzuki et al.2006 unpublished
		Uncultured bifidobacterium from human faeces	GQ896587	99.0	Waddington et al. 2009; unpublished
		Bifidobacterium ruminantium	D86197	98.8	Miyake et al. 1996;

					unpublished
Clone 9	637	Uncultured *Bifidobacterium* sp. from human faeces	AM238028	100	[207]
		Bifidobacterium adolescentis isolate from human faeces	AY305304	97.1	Louis P. 2003 unpublished
		Bifidobacterium adolescentis^{T}	AP009256	97.3	Suzuki et al 2006 unpublished
Clone 19	639	Unclassified *Ruminococcaceae* from human faeces	EU201646	100	[196]
		Eubacterium siraeum	EU266550	96.0	[208],
		Ruminococcus callidus^{T}	L76596	88.3	Wilson et Duncan 1996, unpublished
Clone 20	633	Uncultured *Sporacetigenium* from chicken litter	DQ250647		[209]
		Unclassified *Peptostreptococcaceae* sp. from human faeces	EU762133	96.1	[54]
		Clostridium irregulare^{T}	X73447	95.9	Lawson et al. 1993, unpublished

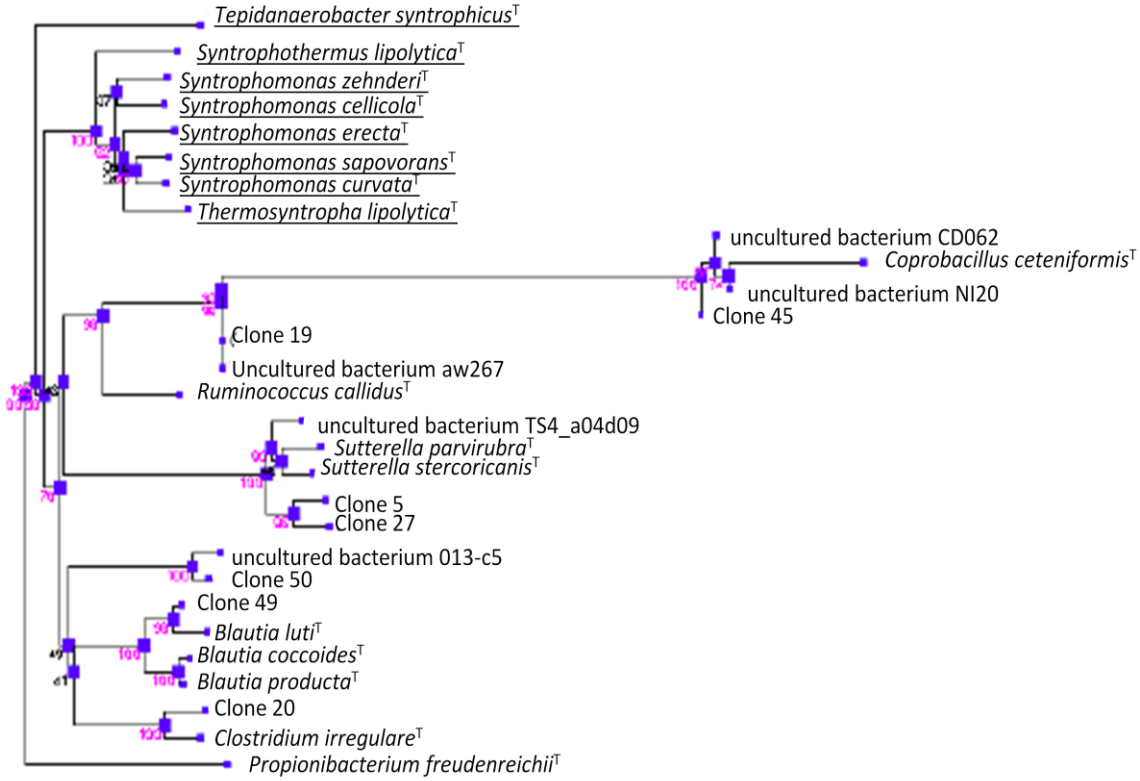


Figure 24: phylogenetic tree displaying the sequences identified from the heavy fraction of the labelled sample after four hours incubation in comparison with known butyrate-consuming bacteria (underlined).

7.4 Discussion

A faecal sample was pulsed with 13C-labelled butyrate *in vitro* and incubated under anaerobic conditions at 37˚C.

Previous studies have found that butyrate consumption occurs only in syntrophic consortia of acetogenic bacteria and methanogenic archaea [185-187]. Thus destructive sampling was chosen in order not to disrupt the microstructure of the sample. The colonic environment is anaerobic and the vast majority of its inhabitants are strictly anaerobic. The sampling procedure might have had an influence on the

metabolic capacity of the stool microbiota, since the sample was exposed to aerobic conditions for approximately 15 minutes before reaching the anaerobic chamber.

The increase of SCFA level in the sample, most prominently acetate and propionate indicated that at least the enzymes involved in their synthesis were still active throughout the incubation. A further indication for active bacterial metabolism is the fact that the yield of RNA was constant 30-45 µg g^{-1} in all samples throughout the experiment.

Most applications of stable isotope probing are limited to assimilatory processes. Bacterial removal of butyrate from the faecal stream has –to our knowledge- not yet been described in the human intestinal tract.

The GC-FID analysis of SCFAs showed a slight increase in butyrate concentrations during the first four hours of the incubation period and stable concentrations thereafter. This finding indicated that either no butyrate was consumed or that its production exceeded its consumption during the first four hours after butyrate pulse before reaching equilibrium. The flattening of the butyrate production curve after four hours might indicate that butyrate production rate decreases due to for example depletion of substrates and inhibition through increased pH. Under physiological conditions SCFAs are constantly removed from the gut lumen by the gut epithelium, which could not be mimicked in this *in vitro* experiment. Inhibition of colonic *Bacteroides* spp. and *E.coli* by decreased pH has previously been shown [210], leading to enrichment of acid-tolerant species able to exploit the acidic niche created during the course of the experiment. Accumulation of butyrate itself is less likely to inhibit its production, since in the samples pulsed with an excess of butyrate this SCFA was still produced too until four hour after the pulse. On the other hand, the flattening of the butyrate production curve might also indicate that after four hours the consumption and production of butyrate reached equilibrium.

The assessment of heavy carbon in the extracted RNA did not show enrichment of ^{13}C. For isotope ratio analysis 5 µg RNA were cut with sucrose in order to reach the

minimum amount of 25µg carbon necessary. If butyrate consumption was only a minor process within the GI microbiota, only a small amount of RNA would have been labelled. Heavy label might have been diluted to such an extent as to remain undetected after cutting with sucrose.

In the heavy butyrate pulsed samples, peak RNA abundances were seen in the high density fractions and in the light density fractions. The abundance pattern of RNA in the individual fractions suggested presence of heavy RNA in fractions with density 1.79 to 1.82 g ml^{-1}. RNA fractionation of the control samples yielded a continuous increase of RNA abundance with decreasing density for the H2O control samples, and ambiguous RNA abundance in ^{12}C-pulsed fractions. Some RNA was distributed down the entire gradient in all samples, thus making an unambiguous identification of heavy label incorporation difficult.

DGGE bandpatterns of the heavy pulse sample after 4 hours showed enrichment of some bands in the heavy fractions nourishing the assumption that some heavy butyrate might have been incorporated into bacterial RNA. DGGE bandpatterns of control samples showed highly similar bandpatterns in all fractions.

To see which sequences were enriched in the heavy fractions, clone libraries were constructed and selected clones sequenced.

Sequence 1 (clone 45) was classified as a member of the family *Erysipelotrichaceae*. Its closest relative (99.2% similarity) was bacterium AY916175 previously found in a faecal sample [54]. The *Erysipelotrichaceae* are capable of forming filaments, immobile ad non-sporulating. Members of this family are facultative anaerobic, chemoautotrophic, straight or slightly curved bacilli. *Erysipelotrichaceae* sp. have been described in various environments such as sludge, poultry, pigs, emus, the poultry red mite and other animals [211]. In the pig, some *Erysipelothrix* spp. are pathogenic and cause large economic losses [212].

Coprobacillus cateniformis-related species of the class *Erysipelotrichi* and the family *Erysipelotrichaceae* have been described as ubiquitous in human faeces [213].

Coprobacillus cateniformis has a G+C-content of 32.1 to 33.1 mol% and is a non-spore forming rod [214].

Sequence 2 (cut1) was cut out and found to be 99.2% similar to bacterium EU775497 from *Gorilla gorilla* faeces and to DQ801012 from human faeces. Due to the short length of cut out sequences this sequence was analysed in FASTA and BLASTn and not applying the ribosomal database project tools. However, EU775497 was classified as *Clostridiales*, *Ruminococcacaea* sp.

Several *Ruminococcaceae* spp. have been described to ferment cellulose in the gastro-intestinal tracts of cattle and humans. *R.hydrogenotrophicus* is able to grow with H_2/CO2 as energy source. A study from Finland identified *Ruminococcus bromii*-like and *Ruminococcus torques*-related sequences as ubiquitous in human faeces [213].

Sequence 3 (clone 31) was identified as *Alistipes* sp. Its closest related type strain is *Alistipes putredinis*^{T}. *Alistipes* spp. have been described as strictly anaerobic, bile-resistant, pigment-producing gram-negative rods [215] frequently prevalent in humans. Similar to bacteria of the *Bacteroides fragilis* group *Alistipes* spp. are resistant to kanamycin, vancomycin and colistin [215].

Sequence 4 (clone49) was found to be 98.1% similar to uncultured bacterium DQ905754 [203] previously found in human faeces. It was classified as *Lachnospiraceae incertae sedis. Lachnospira* spp. and are known to ferment pectin in cattle and sheep.

Sequence 6 (clone 27,8) was identified as a *Betaproteobacterium* of the genus *Sutterella*. Its closest relatives (99.7% and 99.5% similarity) FJ719241 and FJ031979 have previously been found in dog and cat faeces. FJ454832 with a similarity to sequence 7 of 98.9% was found in human faeces [202]. *Sutterella* spp. have previously been found in humans and dogs [216]. *Sutterella* spp. are described to be anaerobic, Gram negativ microbes that are resistant to bile, inactive on sugars and capable of reducing nitrate and nitrite.

Sequence 7 (clones 26, 50) was classified as a *Clostridiales* sp.

The sequences related to *Ruminococcaceae*, comprising also the genus *Lachnospira* have been grouped according to phylogenetic analysis into *Clostridium* cluster XIVa [188]. This suprageneric cluster consists of a phenotypically heterogeneous collection of organisms. Representatives of *Clostridium* cluster XIVa have beed described as producers of butyrate, but have not yet been associated with consumption of this short chain fatty acid before. *Eubacterium siraeum*, matching next to clone 19, has been described to possess cellobiose 2-epimerase, catalyzing the reversible epimerization of cellobiose to 4-O-beta-D-glucopyranosyl-D-mannose [217]. In addition to cello-oliogosaccharides, cellobiose 2-epimerase-like hypothetical proteins are suggested to convert lactose to epilactose [217]. Consumption of butyric acid has previously been described for *Clostridium* cluster III and IV.

Figure 7 shows a phylogenetic tree comparing the cloned sequences with known syntrophic bacteria (underlined). It became apparent that the clones from this study did not form a distinct group, wheras most of the reportedly syntrophic bacteria formed a cluster according to their 16S rRNA sequence similarity. *Tepodanaerobacter syntrophicus* is an exception; its sequence divergence from other syntrophic organisms suggests that the ability for syntrophic life might not be limited to a narrowly defined cluster of bacteria. A G+C content comparison with a large gut microbiota dataset consisting of 1226 full-length 16S rDNA sequences showed that – with the exception of bifidobacterium-like sequences- G+C contents of clones corresponded to average and below average GC contents found in the Eckburg dataset of 2005.

False-positive results may have arisen from several sources. Firstly, the migration of RNA in the CsTFA gradient follows a normal distribution. Thus all fractions contain traces of RNA. Apart from the peak of labelled RNA in the heavy fractions non-labelled RNA might have migrated as well in small quantities. In particular G+C-rich sequences such as those related to bifidobacteria might have constituted false-positive findings in the heavy fractions. Furthermore, PCR bias might have possibly distorted the DGGE

fingerprints as it has been previously described for all PCR-based molecular techniques [84].

7.5 conclusion

DGGE bandpattern analysis showed that some sequences were enriched in heavy fractions of a sample pulsed with ^{13}C-butyrate. Control samples pulsed with ^{12}C-butyrate or water lacked such enrichment; they showed uniform bandpatterns in all fractions.

The 13C incorporation could not be unambiguously proven, since isotope ratio analysis of RNA, and the analysis of SCFAs in the samples did not corroborate the results of DGGE analysis. Furthermore, the results showed that during gradient centrifugation and fractionation RNA was distributed down the entire gradient, thus weakening the evidence from the DGGE experiments.

This pilot study generated some evidence that butyrate consumption took place in the GI communities assessed, but further experiments are necessary to strengthen this interpretation.

No assumptions can be made from this experiment to which extent the gastro-intestinal microbiota contributes to butyrate removal from the faecal stream since no labelled metabolites were analysed. Butyrate consumption is likely to be a minor process compared to the large substrate turnover rate of the enormous diversity and abundance of colonic microbes.

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9 Publications

9.1 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.

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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 yrs) and young volunteers (n=17, 18-31 yrs).

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.

INTRODUCTION

It is estimated that between 60-80 % 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 appears to be a 'core microbiota' of approximately 300 phylotypes which is quite resilient to external influences (Dore 2007). In addition to this core group, there are also 'passengers' or transients, sometimes in great numbers, sometimes below detection limit (Favier *et al.* 2003), and members that are unique to some individual (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 (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 *et al.* 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 aging, 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.

MATERIAL AND METHODS

Probands

Seventeen institutionalized elderly aged 86 ± 8 years, BMI 21.75 $\pm$ 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 $\pm$ 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.

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 sec 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, *Bacteroides thetaiotaomicron* DSM 2079^T, *Bifidobacterium longum* DSM20219^T, *B.longum* DSM 20211, *B.pseudolongum* DSM 20099 and *B.thermophilus* DSM 20210 were anaerobically cultivated on blood agar, *E.coli* IMBH 252/07 and clones were 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 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.

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 1.5mM $MgCl_2$. Bovine serum albumin (10mg/ml, Fermentas) was added to a final concentration of 400μg/ml, primer concentration in the reaction volume was 0.5μM. Amplifications were carried out in a Robocycler (Stratagene).

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 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/).

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 analyse 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, *Bacteroides thetaiotaomicron* DSM 2079^T, *Bifidobacterium 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 *Faecalibacterium prausnitzii*^{T}). The reference marker for *Bifidobacteria* specific DGGE analysis consisted of *Enterococcus faecium* DSM 20477^T, *E.coli* IMBH 252/07, *Bifidobacterium 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 *Bacteroides thetaiotaomicron* DSM 2079^T, fecal clones Bt 17 and Bt11 and *Enterococcus 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 *Faecalibacterium prausnitzii*^{T}, 92.1% and 92.9% similarity with *Eubacterium desmolans*^{T} and 95.9% similarity with *Subdoligranulum variabile*^{T}.

TaqMan RTQ-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 3min and 45 cycles of 95°/60°C for 15/ 45 sec. The amplification program for clostridium cluster IV was: denaturation at 95° for 5 min and 45 cycles at 95°/ 55°C for 30/ 45 sec.

DNA of *Bacteroides thetaiotaomicron*^{T} and *Bifidobacterium 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 estimated using the slope of the standard curve and the formula $E=10^{(-1/slope)}$ -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 (% 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.

Statistical analysis.

Food frequency data were analyzed based on Chi-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 $1-\sum ((p_i)^2)$, 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.

RESULTS

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 (Chi^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).

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 faeces compared to control (figure 1). *Bacteroides* were found to represent a larger percentage than *Bifidobacteria* in all samples (figure 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 (figure 1) in their fecal microbiota than young volunteers (p=0.036).

DGGE bandpattern analysis

DGGE fingerprinting with primer pair 341GC-518, which amplified the total microbial community, showed high inter-individual variations (figure 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 figure 2, occurred significantly less frequently in elderly probands. Shannon and Simpson indices of diversity were significantly lower for elderly than control (figure 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 (figure 4) along the first two principal components. Cluster analysis could separate the fingerprints of young and elderly (figure 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.

Bifidobacteria

An average of thirteen 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 (figure 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.

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 (figure 3) showed a tendency to be lower for elderly than for control. PCA results suggest a tendency for less *Bacteroides* diversity with ageing (figure 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.

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 (figure 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 *Faecalibacterium 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 *Subdoligranulum variabile*^{T}, and 97.9%/ 98% similarity to uncultured bacterium DQ793301).

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

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 analysed, 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 aging (Woodmansey et al. 2007, 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 *Faecalibacterium prausnitzii*, a member of the *Clostridium* cluster IV which decreased in our aged group. However several studies (Hopkins et al. 2002, Woodmansey et al. 2007 and 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 et al. 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.

CONCLUSION

Studies comparing elderly and young volunteer microbiota with PCR-DGGE fingerprinting and qPCR 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.

Tables:

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 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 to 1107.

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	GTGCTGCAGAGAGTTTGATCCT GGCTCAG	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	see table 1	55	
	sg-Clep-R			

Figures

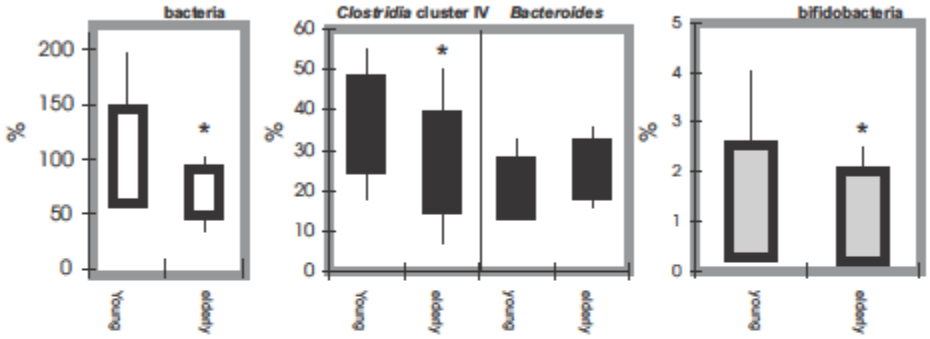

Figure 1: Proportions of *Bacteroides*, *Bifidobacteria* and *Clostridium* cluster IV of the total bacterial load in stool samples of institutionalized elderly and young healthy volunteers. Total amount of bacteria is depicted relative to the mean counts for healthy young young.

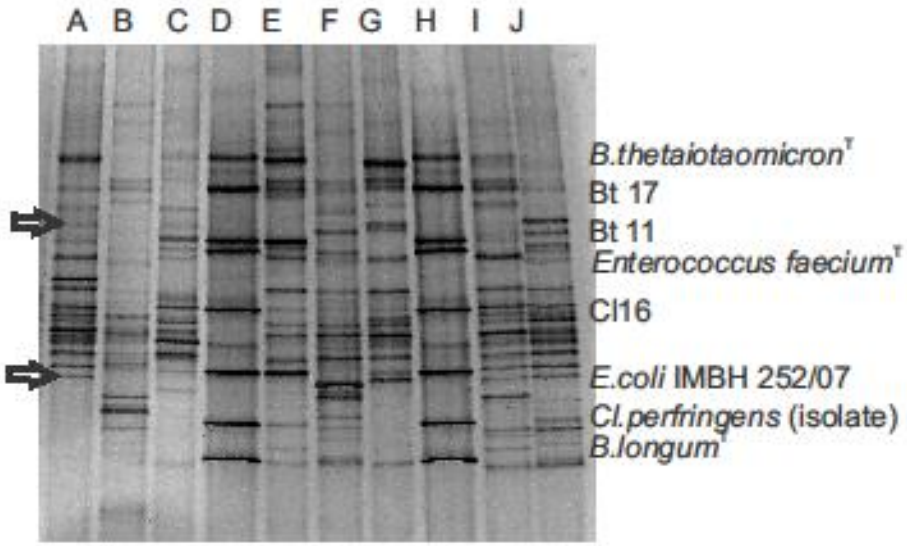

Figure 2: PCR-DGGE bandpattern of 16S rRNA coding regions of dominant bacteria amplified with primer pair 341GC-518. Bands that were observed more frequently in

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

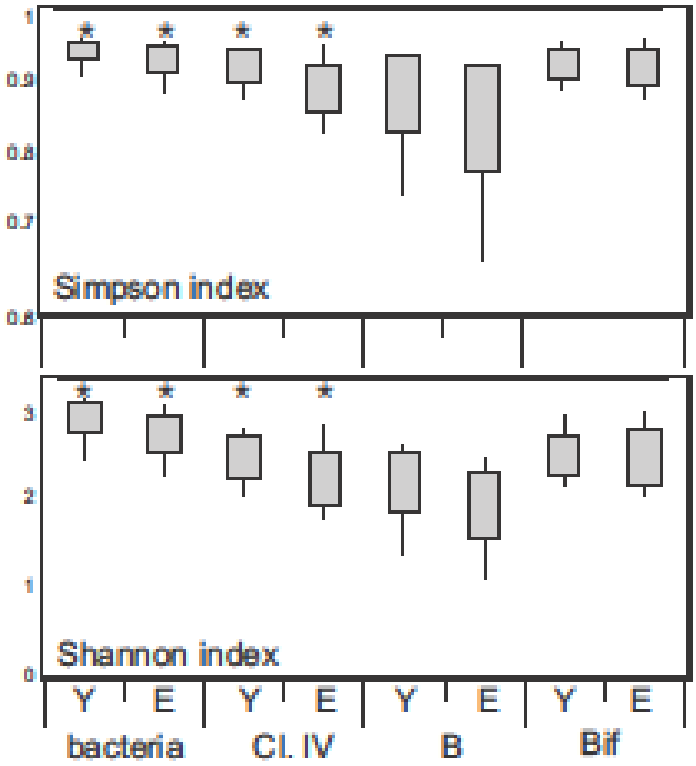

Figure 3: Diversity indices derived from DGGE fingerprinting of 16S rRNA coding regions.

Y, young; E, elderly; Cl.IV, *Clostridium* cluster IV; B, *Bacteroides*, Bif, bifidobacteria; *, $p < 0.05$.

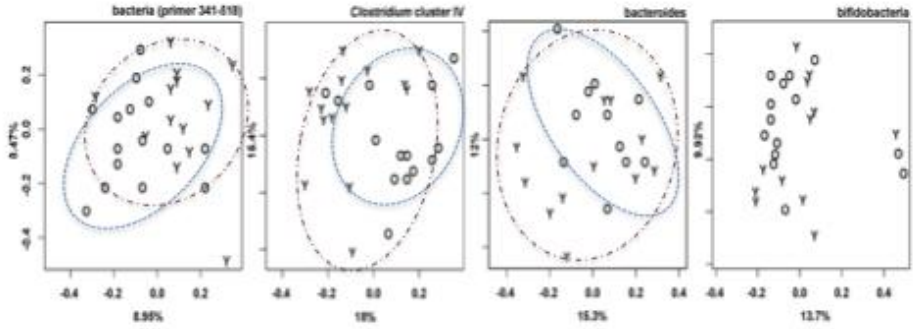

Figure 4: PCA of DGGE fingerprints of 16S rRNA coding regions of dominant bacteria in fecal samples.

Y, young; O, elderly

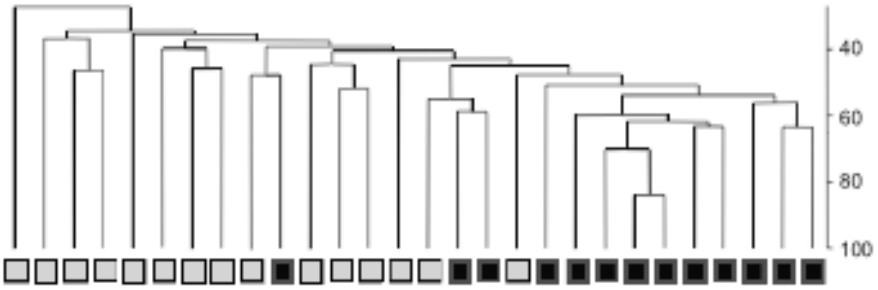

Figure 5: UPMGA dendrogram showing clustering (Dice) based on the similarities of DGGE fingerprints of dominant bacteria obtained with primer pair 341GC-518.

□ elderly; ■ young

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9.2 Characterization of Bacteria, Clostridia, Bacteroides in faeces of vegetarians using qPCR and PCR-DGGE fingerprinting

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Abstract

Background/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.

Methods: Bacterial abundances were measured in faecal samples of 15 vegetarians and 14 omnivores using quantitative PCR (qPCR). Diversity was assessed with PCR-DGGE fingerprinting, PCA (principal components analysis) and Shannon diversity index.

Results: Vegetarians had 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 %), 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: Vegetarian diet affects the intestinal microbiota, especially 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.

Introduction

During the last few decades, vegetarian diets that are frequently associated with little or no animal protein, low fat and high fibre content have known a growing popularity in the Western world and this trend is likely to continue in the future [1, 2]. 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 [1]. Different lifestyle factors and diets were shown to have a significant impact on the faecal microbiota [3-6].

The highly diverse microbiota of the human gastrointestinal tract has been associated with colorectal cancer [7], inflammatory bowel diseases [8, 9], obesity [10], metabolic syndrome [11] and atopic diseases [12]. Two of the most predominant subpopulations in the human faecal microbiota are the *Clostridium* cluster IV (*Clostridium leptum* subgroup) and *Bacteroides* [13-15]. Some members of these populations are fibrolytic and produce butyrate and propionate [16, 17]. The metabolic activities of these organisms have a significant influence on colonic health as butyrate is known as the major energy source for colonocytes [16] and as an epigenetic regulator of gene expression in colonic epithelium [18]. Epigenetic modifications have also been implicated in changes of gene expression associated with vegetarian diet [19, 20].

Another important subgroup of the human microbiota is that of the *Bifidobacteria*. Stimulation of *Bifidobacteria* has previously been shown after prebiotic intervention with inulin and fructo-oligosaccharides [21]. *Bifidobacteria* have been shown to be involved in prevention of atopic disease [22] and prevention of obesity and insulin resistance *via* enhanced barrier function of the gut epithelium [23].

To our knowledge, only one investigation [24] of vegetarian microbiota has been performed with molecular methods. This analysis of a single individual revealed that *Clostridium* cluster XIVa, *Clostridium* cluster IV, *Clostridium* cluster XVIII were the major components of the vegetarian gut microbiota [24].

In the present study, we applied qPCR and PCR-DGGE fingerprinting to investigate the dominant microbiota in 14 young omnivores and 15 vegetarians assessing *Clostridium* cluster IV, *Bacteroides* and *Bifidobacteria.* Analyses of faecal samples have been shown to reflect adequate important changes [25].

Material and Methods

Participants

Twenty-nine healthy young individuals, 15 vegetarians (aged 19-34 years, BMI 22.06 $\pm$ 3.82) and 14 omnivores (aged 21-31 years, BMI 21.02 $\pm$ 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, pre- and probiotics three months prior to sampling. All trial subjects agreed to participate in the study and gave their informed consent.

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 manufacturers' protocol with minor modifications [26, 27] and immediately stored at -20°C.

TaqMan qPCR

Bacterial 16S rRNA was quantified by TaqMan qPCR using previously published primers and probes (table 1). TaqMan Probe (Clept-P) for *Clostridium* cluster IV was designed with CLC DNA Workbench (http://www.clcbio.com). DNA of *Bacteroides thetaiotaomicron*^{T} and *Bifidobacterium longum*^{T}, 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 using Rotor-Gene 3000 calculation software (Corbett operator manual) and Excel.

PCR, DGGE fingerprinting

16S rRNA coding regions were amplified using a ready-to-use mastermix (Promega) in a Robocycler (Stratagene). Group specific primers (table 2) were applied at the temperatures indicated in table 2. 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 [28] with a linear gradient of 25-65% for *Bacteria*, 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.

Statistical analysis

We analyzed qPCR results in Excel applying F-test and Student's T-test. Food frequency data and interesting bands of DGGE fingerprints were analyzed based on Chi-square approximation as implemented in SPSS15. PCR-DGGE band comparison tables were created in GelComparII (www.applied-maths.com) and analyzed with principal component analysis (PCA) using the default settings in 'R-software environment for statistical computing (www.r-project.org) until 100% variance explained. The plots show the transformed data with the first two principal components as x- and y- axis. Shannon diversity indices 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. All tests were double sided, $p<0.05$ was considered as significant.

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 (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.

Relative quantitation

Bifidobacteria, members of *Clostridium* cluster IV and *Bacteroides* were quantified as percentage of the total bacterial DNA (figure 1). 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 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 *Bifidobacteria* in vegetarians (1.52 ± 1.29 %) was unchanged to omnivores (1.59 ± 1.73 %). The three vegans did not stand out in the group of vegetarians.

PCR-DGGE quantitative analysis

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. Some grouping was also visible in the dominant *Bacteria* dataset (figure 2). 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. *Bacteroides* bandpatterns were composed of 9.5 ± 2.9 bands, *Clostridium* cluster IV of 12.75 ± 3.37 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 (figure 3) from *Clostridium* cluster IV were more prevalent in omnivores than in vegetarians (Chi^2 Test; $p<0.005$; $p<0.022$). The first sequence was 96.7 % similar to *Faecalibacterium prausnitzii*^{T} and 99.5 % to *Faecalibacterium prausnitzii* AJ270469. The second sequence 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* [29].

Discussion

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 12 % higher counts of bacterial DNA in vegetarians than in omnivores. A decreased transit time of stool correlating with higher bacterial cell mass due to higher dietary fibre intake [30, 31], as well as pre-biotic 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 [32, 33].

Nevertheless, members of *Clostridium* cluster IV have the same ability. We assume that dietary fibre favours 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 [33-35]. In a cross-sectional study of different European populations, Mueller et al. 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 [36]. 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 [7, 10].

Clostridium cluster XIVa has also been discussed to be affected by vegetarian diet in a study with only one patient [24]. We could not find noticeable differences for this subgroup in our samples (using qPCR according to [37, 38], 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 effects of higher meat consumption as discussed for Clostridium cluster IV in general.

Despite of 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. Summarizing, it remains to be determined if these shifts result in

differential metabolite profiles that might in turn affect host metabolism and disease risks.

Acknowledgments

We would like to thank all study participants and Dr.Viviane Klose and Mag.Verity-Ann Sattler of IfA Tulln for their cooperation in the analysis of DGGE fingerprinting.

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

Tables

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

Target organism	Primer and probe	Sequence (5' - 3')	Size (bp)	Conc. (nM)	Reference
Bifidobacterium spp.	Forward primer	GCG TGC TTA ACA CAT GCA AGT C	125	300	
	Reverse primer	CAC CCG TTT CCA GGA GCT ATT		300	
	Probe	(FAM)- TCA CGC ATT ACT CAC CCG TTC GCC -(BHQ-1)		150	[39]
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	[40]
All *Bacteria*	BAC-338-F	ACT CCT ACG GGA GGC AG	468	1000	
	BAC-805-R	GAC TAC CAG GGT ATC TAA		1000	

		TCC			
	BAC-516-P	(FAM)-TGC CAG CAG CCG CGG TAA TAC-(BHQ-1)		200	[41]
Clostridium cluster IV	sg-Clept-F	GCA CAA GCA GTG GAG T	239	400	
	sg-Clept-R3	CTT CCT CCG TTT TGT CAA		400	[37]
	Clept-P^{++}	(FAM)-AGG GTT GCG CTC GTT-(BHQ-1)		200	This study

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

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

Target organism	**Primer pairs**	**Sequence (5'-3')**	**Ann. temp (°C)**	**Reference**
All *Bacteria*	27f	GTGCTGCAGAGAGTTTGATCCTGGCTCAG	57	[42]
	985r	GTAAGGTTCTTCGCGTT	57	[43]
	341f-GC	CCT ACG GGA GGC AGC AG	55	[23]
	518r	ATT ACC GCG GCT GCT GG	55	[44]
Bacteroides	32f	AACGCTAGCTACAGGCTT	56	[45]
	708r	CAATCGGAGTTCTTCGTG	56	[45]
Bifidobacteria	g-BifidF	CTCCTGGAAACGGGTGG	58	[46]
	g-BifidR	GGTGTTCTTCCCGATATCTACA	58	[46]
Clostridium cluster IV	sg-Clept-F-GC	See table 1	55	[47]
	sg-Clept-R3	See table 1		[47]

Figures

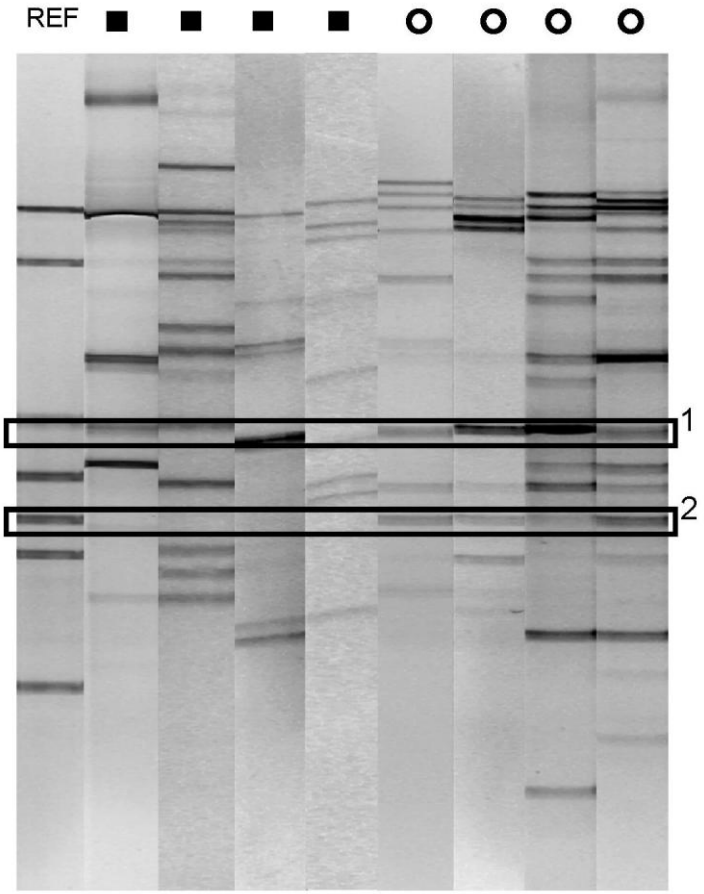

Figure 1: PCR-DGGE bandpatterns of 16S rRNA coding regions of *Clostridium* cluster IV amplified with primer pair sg-Clept-F-GC/sg-Clept-R. Bands that were observed more frequently in omnivores than in vegetarians are indicated with a black box. 1: 94.7% similarity to *Clostridium* sp. BI-114^T and 97.9 % 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 %).

O, omnivores; ■, vegetarians; REF, reference lane

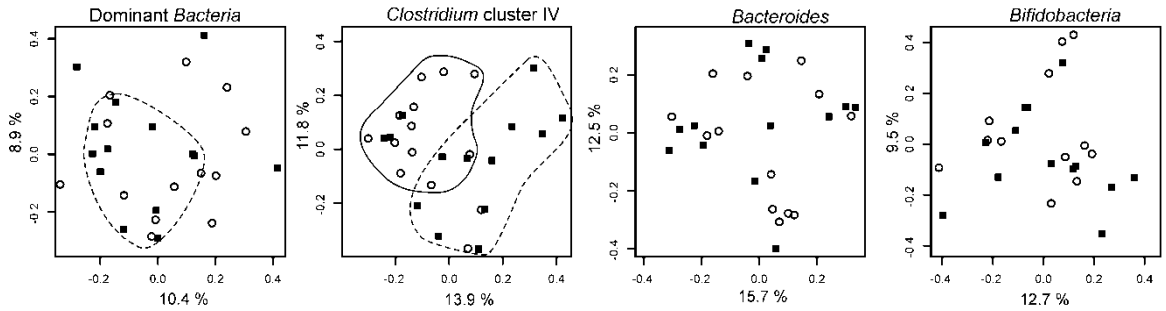

Figure 2: PCA of DGGE fingerprints of 16S rRNA coding regions of dominant *Bacteria*, *Clostridium* cluster IV, *Bacteroides* and *Bifidobacteria* in faecal samples.

O, omnivores; ■, vegetarians

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10 Draft manuscripts

10.1 Quantification of butyryl-CoA CoA transferase genes reveals different butyrate production capacity in individuals of different diet and age.

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Abstract:

Butyrate is a bacterial metabolite of the human gastro-intestinal microbiota with considerable importance for colonic health.

We compared butyryl-CoA CoA transferase gene abundances with a 16S rRNA gene quantification of bacteria, *Clostridium* cluster IV & XIVa in order to assess the butyrate production capacities in human microbiota samples.

Faecal samples of young healthy omnivores (n=15, age: 24 ± 2.5 yrs/BMI 22.68 ± 3.4), vegetarians (n=15, age: 26 ± 5 yrs/ BMI 21.02 ± 2.71) and elderly (n=15, age: 86 ± 8 yrs / BMI 21.75 ± 5.08) were analysed in quantitative PCR (qPCR). Diet and lifestyle was assessed in questionnaire-based interviews.

Elderly had significantly less copies of the butyryl-CoA CoA-transferase gene than omnivores (p=0.014) and vegetarians (p=0.048). The butyrate gene variant related to *Roseburia/E.rectale* spp. was significantly more variable in vegetarians than in elderly. At the same time the *Clostridium* cluster XIVa was more abundant in vegetarians (p=0.049) and omnivores (p=0.0018). Our results indicate that vegetarian as well as omnivore diet has a similar potential to shape a healthy microbiota.

The elderly microbiota was characterized by low butyrate production capacity, possibly contributing to the development of degenerative diseases and anorexia in advanced age.

Introduction

The human gastrointestinal (GI) tract harbours a microbiota, whose number of cells exceeds the human body cells by a factor of 10 or 100 (1). Recent evidence suggests that 1000- 1150 different species are capable of living in the gut ecosystem (2). An individual harbours at least 160 species (2), with great inter-individual variations in species diversity and evenness.

It has previously been suggested that the microbiota composition is influenced by diet (3) (4) (5), health status (6) (7), age (8) (9) as well as genetic factors (10).The GI-microbiota produces short chain fatty acids (SCFAs), mainly acetate, propionate and butyrate, as intermediates and end products of the degradation of dietary compounds. Acetate and propionate are metabolites for gluconeogenesis in the liver (11). Butyrate is the major energy source for colonocytes and is known to regulate various processes in the human distal gut.

Butyrate has been of particular interest due to its anticancerogenic and anti-inflammatory potential (12), effect on the intestinal barrier (13), visceral pain (14) role in satiety (15) and oxidative stress (16). Butyrate induces apoptosis in colorectal tumour cell lines, reduces metastasis, and protects from genotoxic carcinogens by enhancing expression of phase II detoxification enzymes (17).

Two of the most important groups of butyrate producers appear to be *Faecalibacterium prausnitzii* from the *Clostridium leptum* cluster (or *Clostridium* cluster IV), and the *Eubacterium rectale/Rosebura* spp. which belong to the *Clostridium coccoides* cluster (or *Clostridium* cluster XIVa). The distribution of butyrate production among firmicute bacteria is uneven, and the abundant clusters IV and XIVa indicate both producers and nonproducers of butyrate (18).

Faecalibacerium praunsnitzii is a net acetate utilizer able to hydrolyse starch, inulin, fructose and oligofructose. Strains of *F.prausnitzii* differ in their ability to utilize cellobiose, maltose and melezitose (18). Within the genus *Roseburia* the ability to utilize polysaccharides such as amylopectin, inulin (cichory), and xylan in pure cultures

is widespread, whereas some strains appear to survive by metabolic cross-feeding (19). In spite of their ability to utilize polysaccharides in pure culture, *Roseburia* spp. have been described to compete poorly for these substrates in mixed culture fermentor systems (19).

In human feeding trials, isolated dietary compounds have been shown to promote growth of butyrate producers (20, 32). For example, the consumption of inulin significantly stimulated growth of *F.prausnitzii* The abundance of *Roseburia* spp. in response to inulin supplementation increased in those individuals who harboured *Roseburia inulinovorans*, otherwise decreased (20).

Butyrate is readily taken up by the gut mucosa, so that faecal levels give little information on the butyrate producing capacity of the gut microbiota. Therefore, a functionally based approach was suggested for the enumeration of butyrate-producing bacteria (21) targeting the butyryl-CoA CoA transferase gene. This gene was described to be well conserved phylogenetically (21). Furthermore, the butyryl-CoA CoA transferase route, using acetate as a co-substrate, was suggested to be the most important route for butyrate production in the gut ecosystem (22). Alternative routes for the final steps of butyrate sysnthesis in anaerobic bacteria occur via butyrate kinase or phosphotransbutyrylase that were found to occur in only a minority of bacteria (23) in the human GI tract.

To our knowledge, it has not yet been described if complex dietary behaviours or other epidemiologically defined host factors have a measurable influence on butyrate production. We compared butyryl-CoA CoA transferase gene abundances in young healthy omnivores, vegetarians and institutionalized elderly by real-time PCR together with a 16S rRNA gene quantification of *Clostridium* cluster IV & XIVa.

Materials and Methods

Study subjects:

Group of individuals and sample material used in this study was the same as that used by Zwielehner et al. (2009) and Liszt et al. (2009) (24, 25). The geriatric group (elderly) based on 15 institutionalized subjects (age: 86 ± 8 yrs / BMI 21.75 ± 5.08) from a geriatric department in Vienna. The vegetarian diet group (vegetarian) consisted of 15 young healthy subjects with a vegetarian (age: 26 ± 5 yrs/ BMI 21.02 ± 2.71). Seventeen young healthy subjects (age: 24 ± 2.5 yrs/BMI 22.68 ± 3.4) with a Central European diet formed the mixed diet group (omnivores).

All volunteers were interviewed using a questionnaire assessing: age, gender, body height, weight, individual health status (including chronic or acute diseases and blood lipid levels), life-style aspects such as physical activity and dietary habits. Group populations were gender balanced. Only non-pregnants and probands without digestive/gastrointestinal symptoms and no antibiotic or chemotherapeutic treatment three months prior to sampling participated on this study. All probands agreed to participate in the study and gave their informed consent. Approval was obtained from the viennese Human Ethics committee (3., Thomas-Klestil-Platz 8/2).

Quantification of specific metabolic genes and 16S rRNA genes by real-time PCR (qPCR)

The butyryl-CoA CoA transferase gene was amplified with degenerate primers BCoATscrF 5'- GCIGAICATTTCACITGGAAYWSITGGCAYATG –3' and BCoATscrR 5'-CCTGCCTTTGCAATRTCIACRAANGC –3' as described elsewhere (20) on a Rotor-Gene 3000A (QIAGEN) using the SensiMix Sybr (QIAGEN). Absolute quantification was performed using a purified PCR product of know concentration and known length. Amplification with primer pair BcoATscr resulted in clearly distinguishable peaks. Those peaks were grouped according to their melt temperatures as previously described (23). These different peaks (presented as dF/dT) were attributed to *Eubacterium hallii* & SS2/1 (83.2-84.54°C), *Roseburia*/*Eubacterium rectale* spp. (85.84-87.07°C) and *Faecalibacterium prausnitzii* (88.08-89.53°C) (23). *Clostridium* clusters IV and XIVa were quantified on a Rotor-Gene 3000A (QIAGEN) using the SensiMix Taq

(QIAGEN). Dominant bacteria were amplified as previously described (26). The *Clostridium leptum* cluster was quantified using CleptF 5'-gcacaagcagtggagt-3', CleptR 5'-cttcctccgttttgtcaa-3' (27) and CleptP 5'- (FAM)-AGG GTT GCG CTC GTT-(BHQ-1)-3' (24). The *Clostridium coccoides* cluster was amplified using 195F 5'-GCAGTGGGGAATATTGCA-3' (28), Ccocc-F-P 5'- (FAM)- AAATGACGGTACCTGACTAA-(BHQ-1)-3' and Ccocc-R 5'- CTTTGAGTTTCATTCTTGCGAA-3' (27). Amplification programs included an initial denaturation at 95°C for 10 min followed by 40 cycles consisting of denaturation at 95°C for 30 s, annealing at 53°C (butyryl-CoA CoA transferase gene) 55°C (bacteria, *Clostridium* cluster IV), 56°C (*Clostridium* cluster XIVa) for 30 s and extension at 72°C for 50 s. Samples were quantified using a cloned sequence with known concentration in the case of *Clostridium* cluster IV and *Blautia coccoides*^{T} pure culture for cluster XIVa. Clone libraries obtained with primer pair 195F- CcoccR and CleptF-CleptR were analysed to confirm the specificity of the chosen primers.

Data analysis

All statistical analyses were calculated using Origin Pro (www.originlab.com). The F-test was used to test equality of variances. Normality was determined applying the Kolmogorov-Smirnov test. ANOVA was performed using the nonparametric Kruskal-Wallis test. Normally distributed data series were pairwise compared using the Student's t-test. Spearman's rank correlation coefficient was determined between the abundance of clostridial cluster IV and XIVa and the corresponding butyrate gene melt peaks (88.08-89.53°C) and (85.84 – 87.07°C, 83.2-84.54°C).

Results

Dietary analysis

Exercise levels (vegetarians to omnivores) were comparable. Analysis of the participants' dietary habits indicated similar consumption in respect of fruit and milk products in the individual groups. Vegetarians stated significantly more frequent (Chi^2

Test; $p<0.027$) consumption of vegetables than omnivores but similar consumption patterns of carbohydrates, including whole grain products several times a week. The elderly group stated significantly more frequent (Chi^2 Test; $p<0.04$) consumption of meat and similar vegetable consumption than young omnivores. The elderly did not consume any whole grain products. Because many well known SCFA systhesising bacteria belong to the clostridial clusters IV and XIVa we analysed those bacterial subgroups relative to total bacterial DNA.

Relative Quantitation

Vegetarians had 12 ± 62% more bacterial DNA than omnivores. Elderly had 31 ± 21% less bacterial DNA than young omnivores. These differences were not significant due to high individual variations. Many well-known SCFA synthesising bacteria belong to *Clostridium* clusters IV and XIVa. These clostridial clusters XIVa und IV were quantified relative to total bacterial DNA (figure 1). The *Clostridium* cluster IV was significantly more abundant in omnivores (36.3 ± 11.2%) than in elderly (27 ± 11.7%, p=0.036). Vegetarians harboured *Clostridium* cluster IV at 31.86 ± 17.00%. The *Clostridium cluster* XIVa was significantly more abundant in omnivores (19.01 ± 6.7%, p=0.0018) and vegetarians (14.52 ± 5.6%, p=0.049) than in elderly (9.89 ± 6.64%).

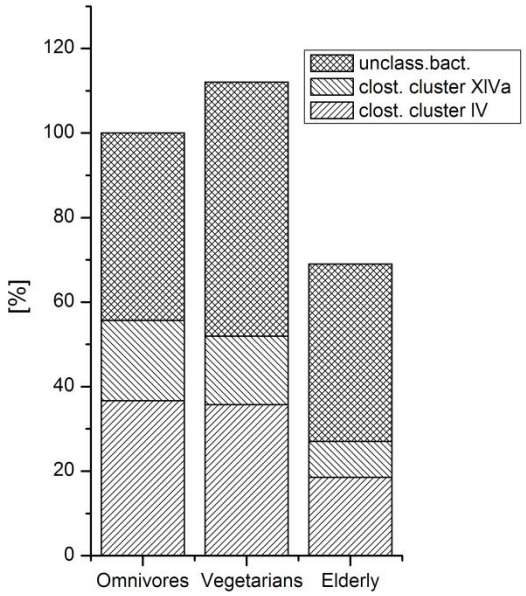

Figure 1: Relative quantities of *Clostridium* cluster IV and XIVa based on group-specific qPCR of 16S rRNA genes.

Detection of butyryl-CoA CoA-transferase genes in faeces by RT-PCR

The butyryl-CoA CoA transferase gene was analysed applying the BCoATscr primer pair [1] (figure 2). Elderly (11.07 ± 14.84ng/µl) had significantly less copies of the butyryl-CoA CoA-transferase gene than omnivores (38.66 ± 35.04 ng/µl, p=0.014) and vegetarians (106.89 ± 164.41ng/µl, p=0.048).

Given even disribution of the butyryl-CoA CoA transferase gene among the gastro-intestinal bacteria, those values would correlate with the overall abundance of bacteria on an individual level. Spearman's rank correlation test showed that the abundance of the butyryl-CoA CoA transferase gene did not correlate with bacterial abundance.

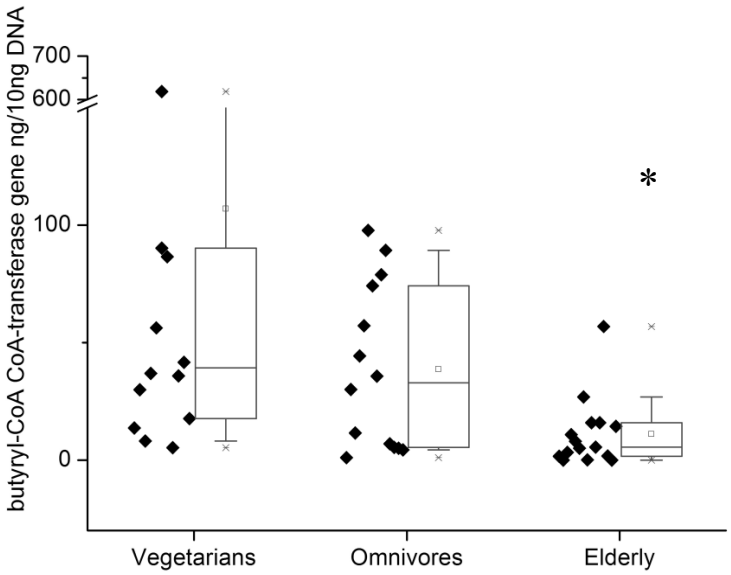

Figure 2: Quantification of the butyryl-CoA CoA transferase gene in faecal samples. Asterisk indicates a significant difference at the 95% confidence interval, rectangular marks represent mean levels; medians are indicated with a line.

To evaluate diversity and abundance of different variants of the butyryl-CoA CoA transferase gene melt curves from faecal samples (figure 3) were divided into three areas as described by Louis et al. (20). Peaks broadly represent bacteria related to *Eubacterium hallii* and SS2/1 (83.2 – 84.54°C), *Roseburia*/*E.rectale* spp. (85.84 – 87.07°C) and *F.prausnitzii* (88.08 – 89.53°C).

The *Eubacterium hallii* / SS2/1 melt peak tended to be higher in vegetarians (p=0.08) and omnivores (p=0.09) than in elderly.

In both omnivores and vegetarians 12 out of the 15 participants harboured a *E.rectale/Roseburia* spp. melt peak, whereas only 6 elderly did. ANOVA showed that the abundance of this particular butyrate gene differed significantly between vegetarians and elderly (p=0.04).

One of the most abundant representatives of *Clostridium* cluster IV in human faeces is *F.prausnitzii.* The abundance of the melt peak attributed to *F.prausnitzii* did not differ in vegetarians, omnivores and elderly.

To see if the abundance of the butyryl-CoA CoA transferase genes of *E.rectale/Roseburia* spp. correlated with the abundance of *Clostridium* cluster XIVa at an individual level, spearman's rank correlation test was calculated. The same test was used to find out if the abundance of the *F.prausnitzii* butyryl-CoA CoA transferase gene correlated with the abundance of *Clostridium* cluster IV. No correlation between abundance of the phylogenetically conserved butyrate genes with the clostridial clusters could be found.

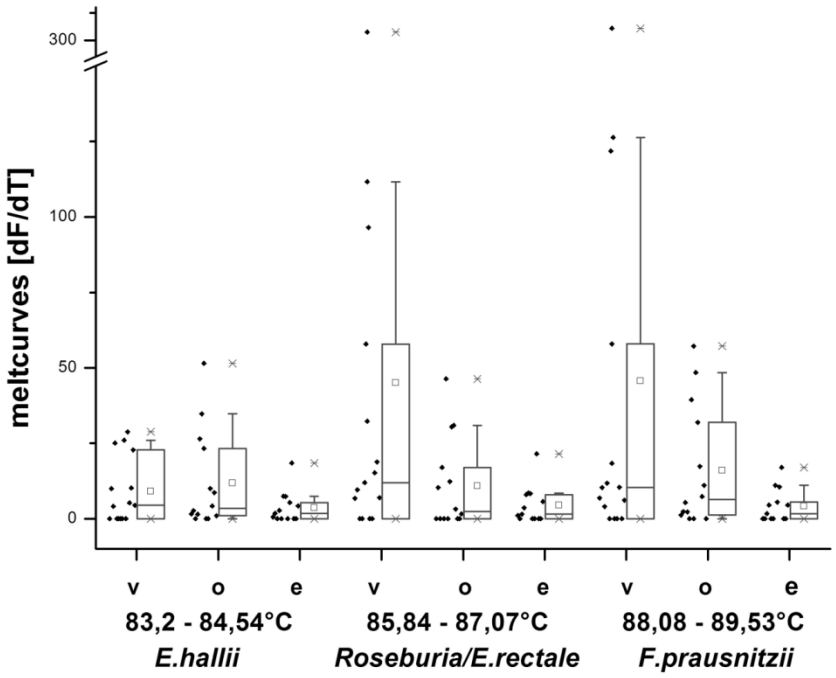

Figure 3: Quantification of butyryl-CoA CoA transferase genes grouped according to their melt curves.

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v, vegetarian; o, omnivore; e, elderly; rectangular marks represent mean levels; medians are indicated with a line.

Discussion

To our knowledge, it has not yet been described if complex dietary behaviours or other epidemiologically defined host factors have a measurable influence on butyrate production. This study asks if elderly, young omnivores and young vegetarians differ in abundances of butyryl-CoA CoA transferase genes and polysaccharide degrading bacteria of the clostridial clusters IV and XIVa. Furthermore, we ask if these functional gene-based and 16S rRNA gene-based abundances correlate with each other.

Young vegetarians and omnivores had significantly more copies of the butyryl-CoA CoA-transferase gene than elderly. Analysis of the overall abundance of bacterial 16S rRNA genes revealed that vegetarians harboured more bacteria than omnivores; lowest numbers of bacteria were found in elderly individuals in spite of great individual variations. On an individual scale butyryl-CoA CoA transferase gene abundance did not correlate with overall bacterial 16S rRNA gene abundance.

In a melt curve analysis three groups of butyryl-CoA CoA transferase genes could be identified. Louis et al. (20) found that melt curve patterns corresponded well with 16S rRNA gene abundances of *E.hallii*/SS2/1, *Roseburia/E.rectale* spp. and *F.prausnitzii*.

The butyrate gene variant related to *Roseburia/E.rectale* spp. was significantly more variable in vegetarians than in elderly. At the same time the *Clostridium* cluster XIVa was more abundant in vegetarians. The abundance of the *Clostridium* cluster XIVa did not correlate with the abundances of the butyrate gene variant related to *Roseburia/E.rectale* spp.

The *Clostridium* cluster IV was significantly more abundant in omnivores than in elderly. One of the most abundant representative of *Clostridium* cluster IV in human faeces is *F.prausnitzii.* The abundance of the melt peak attributed to *F.prausnitzii* did not differ in vegetarians, omnivores and elderly.

The primer pairs used here broadly target the clostridial clusters IV and XIVa. As previously described (18) the abundance of butyrate producers in cluster IV and XIVa is uneven.

Vegetarian diet might have –in some individuals- favoured growth of *Roseburia/E.rectale* spp. that carry the butyryl-CoA CoA transferase gene, without causing an increase of the entire *Clostridium* cluster XIVa.

In other vegetarians, *Clostridium* cluster XIVa occurred at high abundance without a concomitant increase of the butyratyl-CoA CoA transferase gene of *Roseburia*/*E.rectale* spp.

In our study omnivores had a similar potential to harbour members of *Clostridium* cluster IV and XIVa and butyryl-CoA CoA transferase than vegetarians. We conclude that both vegetarian as well as omnivore diet have a similar potential to shape a healthy microbiota.

Our results point out that elderly harbour less *Clostridium* cluster IV and XIVa in already low levels of overall bacterial. Together with lower abundance of the butyryl-CoA CoA transferase gene, our results indicate that the elderly microbiota might be characterized by low butyrate production capacity. Considering the important nutritive, anti-inflammatory and anticancerogenic potential of butyrate in the human colon, these findings point out that these microbiota specificities might contribute to the development of degenerative diseases (8) and anorexia in advanced age.

Determining the indigenous microbiota composition might be a suitable strategy to learn if/which dietary intervention with prebiotic fibre might be useful to enhance butyrate production in the colon sustainably. In individuals who do not harbour butyric acid producing species, probiotic supplementation or the administration of coated butyrate capsules might be better alternatives than increased ingestion of dietary fibre.

Physical activity has been described as an important factor for gut motility and intestinal passage time (29). Gut motility and intestinal passage time have been described to be major determinants of bacterial growth in the gut ecosystem (29). In addition to lower physical activity, elderly study participants did not consume any whole grain products, whereas young volunteers did state regular consumption of such fibre-rich products. Further research is required to investigate the possibility of deliberately introducing butyrate-producing species into the human GI-tract via probiotic supplementation.

Assessment of dietary behaviour is affected by bias, because quick and affordable methods based on questionnaires rely on self-reporting (30) and accurate, well maintained food databases.

Quantification of selected biomarkers might be better suited for an individualized assessment of GI-health and microbiota function.

In addition to the possible bias inherent to all PCR based methods, amplification of the 16S rRNA gene can be particularly biased because bacteria can have multiple operons for the 16S rRNA gene (31). Thus, quantification of the butyryl-CoA CoA transferase gene might is a suitable biomarker for butyrate production capacity and GI-health in addition to quantification of the 16S rRNA genes of *Clostridium* cluster IV and XIVa alone.

Our melt curve analysis assumes that the intensity of individual peaks represent the initial proportion of the different butyryl-CoA CoA transferase gene variants. Bias might have come from different amplification efficiencies of the phylogenetically conserved gene variants.

The use of degenerate primers carries a certain risk for unspecific amplification of non-target DNA. As a rough estimation of the accuracy of our amplification, we checked every PCR product in 2% agarose gels where all PCR products gave bands of the expected size.

A more detailed dietary analysis, possibly also including a weighing log of all the food ingested will be necessary in the future to gain further insight into the effects of diet on the GI microbiota. Quantification of the butyryl-CoA CoA transferase gene might be a suitable biomarker for butyrate production capacity together with quantification of the clostridial clusters IV and XIVa.

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10.2 Changes in human fecal microbiota due to chemotherapy analyzed by TaqMan-PCR and PCR-DGGE fingerprinting

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Abstract

We investigated whether chemotherapy ± antibiotics against different kinds of cancer change the gastrointestinal microbiota with special regard to the *genera Bacteroides*, bifidobacteria, *Clostridium cluster IV* and *XIVa* and the potential appearance of *Clostridium difficile*.

Feces of eleven ambulant patients receiving chemotherapy ± accompanying antibiotics were analyzed before and after the onset of treatment at four time points in comparison to 9 gender-, age- and lifestyle-matched healthy controls. We targeted 16S rRNA genes of bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV* and *XIVa* as well as *C. difficile* with TaqMan qPCR and denaturing gradient gel electrophoresis (DGGE) fingerprinting.

Molecular analysis of the fecal microbiota showed that chemotherapeutic ± antibiotic treatment significantly decreased the abundance of bacteria. After the end of chemotherapeutic cycles the microbiota recovered within a few days sometimes even showing a “rebound-effect”. The chemotherapeutical treatment marginally affected the genus *Bacteroides* while the genera *Clostridium* cluster *IV* and *XIVa* were more sensitive to chemotherapy and antibiotic treatment. DGGE fingerprinting showed decreased diversity of bacteria and *Clostridium* cluster *XIVa* and altered diversity of

Clostridium cluster IV in response to chemotherapy. The incidence of *C. difficile* in two out of eleven subjects was accompanied by a decrease of the genera bifidobacteria and *Clostridium* cluster *IV*.

Despite high individual variations, these results suggest that changes in the human microbiota are a side effect of chemotherapy ± antibiotic treatment. Perturbed microbiota could be one cause for severe gastrointestinal disturbances and therefore may be a target for specific mitigation with safe pre- and probiotics.

Introduction

The intestinal ecosystem constitutes the microbiota that can be pictured as a microbial organ placed within a host organism and involves a dynamic interplay between food, host cells and microbes (Turroni *et al.*, 2008). The microbiota plays several significant roles in the digestion of food, energy regulation, generation of short-chain fatty acids, vitamin synthesis, prevention of colonization by pathogens and protection against cell injury (Tlaskalova-Hogenova *et al.*, 2004; Backhed *et al.*, 2005; De La Cochetiere *et al.*, 2005; Eckburg *et al.*, 2005; Turnbaugh *et al.*, 2009). Moreover, the gut microbiota influences the host by directing intestinal epithelial cell proliferation and differentiation, pH, and the development of the immune system (Turroni *et al.*, 2008). Recent culture-independent molecular studies on healthy individuals have shown that the intestinal microbiota is specific to the host and resistant to modifications over time (De La Cochetiere *et al.*, 2005).

A healthy microbiota contains a balanced composition of many classes of bacteria (Round & Mazmanian, 2009). The fecal microbiota is dominated by the *Clostridium coccoides* group - clostridial cluster XIVa (lately reclassified by Liu et al. as *Blautia coccoides*) the *Clostridium leptum* group - *Clostridium* cluster *IV* and the *Bacteroides* (Liu *et al.*, 2008; Maukonen *et al.*, 2008). All three groups are known to positively affect the gut health through nutrient absorption, production of short chain fatty acids (SCFAs) and epithelial cell maturation (Maukonen *et al.*, 2006; Woodmansey, 2007).

Moreover, the subgroup bifidobacteria seems to be an important part of the GI microbiota, being involved in the prevention of atopic disease, obesity and insulin resistance via enhanced barrier function of the gut epithelium (Zwielehner *et al.*, 2009).

To prevent the invasion of endogenous bacteria from oral cavity and the gastrointestinal tract, three defense mechanisms are considered to be relevant: innate immunity, mechanical mucosal barrier, and colonization resistance (van Vliet *et al.*, 2009). However, chemotherapy and the use of antibiotics damage the rapidly generated mucosal cells of the gastrointestinal tract and disrupt the ecological balance, allowing pathogens such as *Clostridium difficile* to grow (Nyhlen *et al.*, 2002; Guarner & Malagelada, 2003). This bacterium is thought to be the causative agent in up to 20% of antibiotic-associated diarrhea (AAD) cases (Koning *et al.*, 2008). It is evident that the intestinal microbial ecosystem has an important but incompletely defined role in mucosal protection (Croswell *et al.*, 2009).

Mucositis is a major oncological problem, caused by the cytotoxic effects of cancer chemotherapy and radiotherapy. Approximately 40% of patients receiving standard dose chemotherapy and up to 100% of patients receiving high dose chemotherapy and stem cell or bone marrow transplantation suffer from abdominal pain, ulceration, bloating and vomiting (Stringer *et al.*, 2007; Stringer *et al.*, 2009). Although gastrointestinal disturbances (mucositis, diarrhoea and constipation) and immunosuppression are well recognised side-effects of cancer treatment, very little research has been conducted into the underlying mechanisms and the changes in the composition of the microbiota. Because of these changes, nutrient absorption and other intestinal functions involving the microbiota may also be altered (Gibson & Keefe, 2006).

For this reason, we investigated shifts in fecal microbiota of patients receiving cancer chemotherapy with or without antibiotics in comparison to healthy control individuals. We sampled at four time points before or after chemotherapy to study changes in fecal microbiota over the course of time. In this study we aimed to clarify

how chemotherapy agents influence fecal bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV*, *Clostridium* cluster *XIVa* and *C. difficile* using culture-independent methods assessing abundance and diversity. Furthermore, we hypothesize that the human fecal microbiota is resilient in its ability to return to its original composition after cycles of chemotherapy.

Material and Methods

Study participants and study design

Eleven subjects receiving chemotherapy with or without antimicrobial therapy (aged 55 ± 14 years, BMI 28 ± 7) from the Sozialmedizinisches Zentrum Ost in Vienna and nine healthy individuals (aged 55 ± 21 years, BMI 24 ± 5) joined this study. Fecal samples of each ambulant oncology patient were collected at four time points within two weeks before and after the onset of treatment. Two out of eleven patients had never received any chemotherapy before, while the others had a history of chemotherapy. One subject receiving chemotherapy additionally suffered from rheumatism while another subject suffered from diabetes mellitus type II, hypertension and obesity. Anonymous medical records reported types of malignancies as well as chemotherapeutic and antimicrobial treatment. Four individuals suffered from a form of leukaemia, three patients suffered from a form of lymphoma (non-hodgekin). Other malignancies were breast cancer, bladder cancer ovarian arrhenoblastoma and multiple myeloma. Among the chemotherapeutic regimens were bendamustin, bortezomib, cytarabin, dexamethosane, doxorubicin, etoposid, gemcitabine, idarubicin and melphalan. Leukaemia patients furthermore received G-CSF (neupogen) and/or radiated erythrocyte concentrate.

Stool samples of healthy individuals were collected once. Study populations were gender balanced, with 55% females in both oncology patients and healthy controls.

We interviewed all study participants assessing: age; gender; body length; weight; health status (chronic and acute diseases); life-style aspects such as cigarette and

alcohol consumption as well as physical activity. Dietary habits were assessed using a food frequency questionnaire. Exclusion criteria for healthy controls were (a) antimicrobial medication (b) chemotherapeutic treatment and (c) pre- and probiotics at least three months before sample collection.

All subjects gave written informed consent. The ethics committee of Vienna approved the study.

Stool sample processing

After collection, stool samples were brought to the laboratory and immediately stored at -70°C. A 200 mg aliquot of each sample was treated twice for 45 s in a bead-beater (Mini-Beadbeater-8). Thereafter DNA was extracted using the QIAamp® DNA Stool Mini Kit (QIAGEN) following the manufacturer's protocol. Finally we stored the DNA at -20°C until analysis.

Type strains

We used type strains, known to be part of the human gastrointestinal microbiota and cloned sequences, to design a DGGE standard lane marker. Type strains *Bacteroides thetaiotaomicron* DSM 2079^T, *Enterococcus faecium* DSM 20477^T, *Lactobaccilus reuteri* ATCC 55730^T, *Bifidobacterium longum* ssp*. longum* DSM 20097^T, *Escherichia coli* IMBH 252/07 and clones *CL16* and *CC34* (see below) were used creating a comparable standard lane marker for DGGE gels analyzing all bacteria (figure 1).

Clone library

To create a standard lane marker for DGGE analysis and to identify dominant members of the *Clostridium* cluster *XIVa* we constructed a clone library from stool samples. For this purpose PCR products amplified with primers 195-F (Meier *et al.*,

1999) and Ccocc-R (Matsuki *et al.*, 2004) were inserted into a p-GEM Easy Vector (Promega) following the instructions of the manufacturer. Nucleotide sequences were corrected for primer and vector sequences in CodonCodeAligner (www.codoncode.com) and taxonomically identified using the online tools of the ribosomal database project (http:// rdp.cme.msu.edu/). The clone library used for creating a standard lane marker for DGGE analysis of *Clostridium* cluster IV has previously been described (Zwielehner *et al.*, 2009).

Polymerase chain reaction (PCR)

PCR was carried out amplifying 16S rRNA gene sequences from bacteria in fecal samples, type strains and cloned sequences for DGGE analysis as well as for creation of the clone library using group-specific and kingdom-specific primers (table 1). The PCR reaction mixture consisted of ready-to-use mastermix (Promega) with 1.5 mM $MgCl_2$, 500 nM of primers and 2 µl of template DNA. When amplifying fecal samples, bovine serum albumin (Fermentas) was added to a final concentration of 400µg/ml. We used a Robocycler (Stratagene) for all amplifications.

PCR-DGGE-fingerprinting

DGGE was performed as described by Muyzer et. al. (Muyzer *et al.*, 1993). Primer pairs and annealing temperatures to analyze the diversity of (a) bacteria, (b) *Clostridium* cluster *IV* and (c) *Clostridium* cluster *XIVa* are described in table 1.

PCR products were separated by polyacrylamid gels with a denaturing gradient of 30-60% for predominant bacteria, 30-50% for *Clostridium* cluster *IV* and 35-50% for *Clostridium* cluster *XIVa.* Electrophoresis was performed for 9 h at 129 V at 60°C (predominant bacteria), 5 h at 200V at 60°C (*Clostridium* cluster *IV*) and 7 h at 200 V at 60°C (*Clostridium* cluster *XIVa*). We created a standard lane marker for each DGGE analysis assay to ensure reliable gel-to-gel comparison. These standard lane markers

(described above) were loaded in triplicate on each gel to adjust for gradient-variations within gels.

Table: 16S rRNA gene primers used for PCR-DGGE fingerprinting.

Target organism	**Primer pairs**	**Sequence (5'-3')**	**Ann. temp (°C)**	**Reference**
All bacteria	27f	GTGCTGCAGAGAGTTTGATCCTGGCTCAG	57	[103]
	985r	GTAAGGTTCTTCGCGTT	57	[104]
	341f-GC	CCT ACG GGA GGC AGC AG	55	[63]
	518r	ATT ACC GCG GCT GCT GG	55	[91]
Bacteroides	32f	AACGCTAGCTACAGGCTT	56	[97]
	708r	CAATCGGAGTTCTTCGTG	56	[97]
bifidobacteria	g-BifidF	CTCCTGGAAACGGGTGG	58	[105]
	g-BifidR	GGTGTTCTTCCCGATATCTACA	58	[105]
Clostridium cluster IV	sg-Clept-F-GC	GCA CAA GCA GTG GAG T	55	[129]
	sg-Clept-R3	CTT CCT CCG TTT TGT CAA		[129]
Clostridium	CcoccF-	AAATGACGGTACCTGACTAA	55	[129]

cluster XIVa	GC		
	CcoccR	CTTTGAGTTTCATTCTTGCGAA	[129]
GC-clamp		CGCCCGGGGCGCGCCCCGGGCGGC CCGGGGGCACCGGGGGG	[63]

Quantitative TaqMan-PCR

The abundance of bacteria and bacterial subgroups was measured by 16S rRNA gene-targeting TaqMan qPCR. Optimal annealing temperature of the PCR primer pairs and expected product sizes (table 2) were ascertained using a Robocycler (Stratagene), before the TaqMan-assay was performed in a Rotorgene 3000 (Corbett Life Science). We analyzed each sample in duplicate. Amplification reactions were carried out in a total volume of 10µl consisting of 5µl Taq-Man SensiMix DNA Kit (Quantance), 1µl of each primer and Taq-Man-probe (concentrations see table 2) and 10ng of bacterial DNA. Amplification programs included an initial denaturation at 95°C for 10 min followed by 40 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C (*bacteria*, *Clostridium* cluster *IV*), 56°C (*Clostridium* cluster *XIVa*), 58°C (*C. difficile*) or 60 °C (*bacteroides*, Bifidobacteria) for 30 s and extension at 72°C for 50 s.

We used tenfold serial DNA dilutions of type strains *Bacteroides thetaiotaomicron*^{T}, *Bifidobacterium longum ssp. longum*^{T} and *C. difficile* as well as the clones *CL16* and *CC34* and one fecal sample to construct standard curves for comparison of PCR reaction efficiencies among different experiments.

We quantified DNA of *Bacteroides thetaiotaomicron*^{T}, *Bifidobacterium longum ssp. longum*^{T} and *C. difficile*, using the nanodrop method and calculated DNA copiel/µl through mean G+C content of each strain. Quantification of clones *CL16* (*Clostridium leptum* 16) and *CC34* (*Clostridium coccoides* 34) was done determining the numbers of

CFU (colony forming units). Relative percentages of bacterial subgroups were calculated in relation to total rRNA gene copies amplified with primer pair BAC-338-F and BAC-805-R (Yu *et al.*, 2005).

We reviewed sensitivity of PCR reactions with stepwise dilutions of standard curve DNA until we achieved sensitive detection levels of PCR. The specificity was confirmed using non-target DNA.

Table 2: Primers and probes used for relative quantification of bacterial 16S rRNA genes.

Target organism	**Primer and probe**	**Sequence (5' - 3')**	**Size (bp)**	**Conc. (nM)**	**Reference**
Bifidobacterium spp.	Forward primer	GCG TGC TTA ACA CAT GCA AGT C	125	300	
	Reverse primer	CAC CCG TTT CCA GGA GCT ATT		300	
	Probe	(FAM)- TCA CGC ATT ACT CAC CCG TTC GCC -(BHQ-1)		150	[100]
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		100	[101]

		TGC CT-(BHQ-1)			
All bacteria	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	[102]
Clostridium cluster IV	sg-Clept-F	GCA CAA GCA GTG GAG T	239	400	
	sg-Clept-R3	CTT CCT CCG TTT TGT CAA		400	[96]
	Clept-P^{++}	(FAM)-AGG GTT GCG CTC GTT-(BHQ-1)		200	This study
Clostridium cluster XIVa	195F	GCA GTG GGG AAT ATT GCA		500	
	CcoccR	CTT TGA GTT TCA TTC TTG CGA A		500	[96]
	CcoccP	(6-FAM)-AAATGACGGTACCTGAC TAA-(BHQ-1)		150	[96]
Clostridium diffficile	CdiffF	TTG AGC GAT TTA CTT CGG TAA AGA		1000	[100]
	CdiffR	TGT ACT GGC TCA CCT	151	1000	

		TTG ATA TTC A	
	CdiffP	(6-FAM)-CCA CGC GTT ACT CAC CCG TCC G-(BHQ-1)	200

Statistical analysis

Statistical evaluation of differences between groups (chemotherapy and control) and changes within the chemotherapy group (all time points before and after chemotherapy) was carried out using the OriginPro version 8 (OriginLab, Northampton, MA). For two group comparisons of independent ordinal and interval values we used the two-sample-T-test and the nonparametric Mann-Whitney-U-test. For the analysis of related data we used the paired-sample-T-test or the non-parametric Wilcoxon signed-rank test. P values <0.05 were considered statistically significant.

We analyzed PCR-DGGE fingerprints in GelComparII (www.applied-maths.com) and applied principal component analysis (PCA) using the default settings in 'R-software environment for statistical computing' (www.r-project.org) until 100% variance was explained. Shannon and Simpson diversity indices were calculated on quantitative band information 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 index, given as 1 - D, this is $1 - \sum((p_i)^2)$, p_i is the relative frequency of the *i*-th species (Zwielehner *et al.*, 2009). In short, the higher the Simpson (0-1) and Shannon indices are, the higher the diversity.

RESULTS

Dietary aspects

We assessed the participants` dietary habits using a food frequency questionnaire. All study participants (patients and controls) were omnivores and showed similar consumption patterns of liquids, alcohol, fruits, vegetables, grains and milk products. Healthy controls stated more frequent consumption of fruits, whole grain products and alcohol several times a week compared to patients receiving chemotherapy.

Chemotherapeutic treatment with or without antibiotics decreases absolute bacterial numbers in comparison to healthy controls

To investigate whether chemotherapy *with or without* antibiotics changes the human GI microbiota composition in contrast to healthy individuals and over a time period, we investigated absolute numbers and relative percentages of bacterial subgroups. The ambulant patients receiving chemotherapy harboured only 25 ± 22% of the absolute bacterial load in their feces compared to healthy controls and with it less *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV* and *XIVa*. Despite high inter- and intraindividual variations the differences in absolute numbers of bacteria ($p = 0.02$), *Bacteriodes* ($p=0.01$), bifidobacteria ($p=0.001$) and *Clostridium* cluster *XIVa* ($p=0.001$) were statistically significant. Furthermore, oncology patients had less absolute numbers of *Clostridium* cluster *IV*, but not significantly. Abundance of all bacterial subgroups declined after chemotherapeutical intervention often followed by a rebound of bacterial abundance (figure 2).

Subject ON007 shows a sharp decline at time point 4 of all bacteria and bacterial subgroups following blood stem cell transplantation and medical intervention.

Clostridium cluster IV and XIVa show great alterations due to chemotherapeutical interventions, while the geni Bacteroides and* bifidobacteria *seem to be marginally affected

In contrast to absolute numbers in figure 2, figure 3 shows the relative quantification of *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV*, *Clostridium* cluster *XIVa* and *C. difficile* as percentage of total bacterial DNA. The mean proportion of *Bacteroides* in stool samples was 27 ± 12% in chemotherapy patients and 23 ± 12% in healthy individuals. The mean percentage of bifidobacteria in patients was 1.17 ± 1% and 1% ± 0.7% in controls. Patients harboured on average 16 ± 11% of *Clostridium* cluster *IV* and 20 ± 14% of *Clostridium* cluster *XIVa*, while controls harboured 10 ± 7% and 34 ± 22% of clostridial clusters *IV* and *XIVa*.

It can be seen in figure 4A that there is a difference between the oncology and control group with regard to the subgroups *Clostridium* cluster *IV* and *XIVa*. Oncology patients harboured significantly more (p = 0.02) *Clostridium* cluster *IV* and less *Clostridium* cluster *XIVa* (p= 0,057) than healthy controls.

Clostridium cluster IV higher before chemotherapy than after.

Figure 4B illustrates the differences of the fecal microbiota composition before and after chemotherapeutic cycles. The mean percentage of *Clostridium* cluster *IV* before chemotherapy was 22 ± 10% compared to after chemotherapeutic cycles with 14 ± 9% of all analyzed bacteria. Hence, there is a significant difference (p = 0.01). The average amount of *Bacteroides*, bifidobacteria and *Clostridium* cluster *XIVa* were 30 ± 12%, 1.2 ± 1.7% and 22 ± 11% at time points before chemotherapy and 28 ± 12%, 1.6 ±1.5% and 24 ± 17% after chemotherapy.

C. difficile colonization found in individuals receiving chemotherapeutic and antibiotic treatment

To find out whether the chemotherapeutic and antibiotic disruption favours the growth of pathogens, we investigated the abundance of *C. difficile*. Two out of eleven patients (18%) receiving chemotherapy harboured *C. difficile.* Patient ON009 harboured *C. difficile* at four time points investigated with a mean proportion of 0.4 ± 0.7%, showing the highest level (1.22%) at sampling point 3 (highlighted in figure 3), when chemotherapeutic and antibiotic treatment took place. Pathogenic *C. difficile* grew up at time point 3 in patient ON011 (3.90% of all analyzed bacteria, highlighted in figure 3) after chemotherapeutic intervention at time point 2.

PCR-DGGE fingerprinting analysis shows decreased diversity of* bacteria *and Clostridium cluster XIVa in response to medical treatment compared to healthy individuals

DGGE fingerprinting analyses of bacteria, *Clostridium* cluster *IV* and *Clostridium* cluster *XIVa* indicate a highly diverse dataset between individuals and uniqueness of fecal microbiota. The mean numbers of bands per patient receiving chemotherapy were 21.8 ± 5 for bacteria, 9 ± 5 bands for *clostridium* cluster *IV* and of 14.9 ± 7 bands for *clostridium* cluster *XIVa*. DGGE profiles of healthy controls had 23.1 ± 5 bands for bacteria, 9 ± 5 for *clostridium* cluster *IV* and 18.9 ± 7 for *clostridium* cluster *XIVa*. The number of bands as well as Shannon and Simpson diversity indices (figure 5) show a lower diversity of bacteria (figures 5A, 5B) and *Clostridium* cluster *XIVa* (figure 5E, 5F) in oncology patients. In line with increased abundance of cluster *IV* oncology patients had greater diversity of *Clostridium* cluster *IV* according to DGGE fingerprints (figure 5C, 5D). The dataset was subjected to principal component analysis (PCA). PCA analysis extracts underlying components of samples according to their variance. Graphs A and B in figure 6 display PCA of *Clostridium* cluster *IV* fingerprints. While

graph A distinguishes between oncology patients and healthy controls, graph B indicates separation of participants before and after chemotherapy and healthy individuals according to their *Clostridium* cluster *IV* DGGE fingerprints. PCA analysis shows increased variability in the DGGE fingerprints of individuals under therapy compared to healthy controls. Chemotherapy resulted in distinctive clustering of bandpatterns before and after chemotherapy.

Discussion

Chemotherapeutic and antibiotic use has both benefits and risks. Even if chemotherapy and antibiotics are used as life-prolonging measures for critically ill patients and to fight life-threatening infections, both are associated with severe side effects such as mucositis, diarrhoea or constipation. These side effects increase the cost of health services, and are often life-threatening (Stringer *et al.*, 2007). Chemotherapeutic and antibiotic treatment has a detrimental impact on the host microbial ecosystem, which is important for host mucosal protection (Croswell *et al.*, 2009) and thereby increases the risk of infection (van Vliet *et al.*, 2009). Overgrowth of species with potential pathogenicity such as toxigenic *C. difficile* and inflammatory complications are among the most common serious complications of chemotherapy and antibiotic treatment among patients with cancer (Guarner & Malagelada, 2003; van Vliet *et al.*, 2009).

In this study, we investigated how the use of cancer chemotherapy mostly with antibiotic treatment perturbs the fecal microbial ecosystem during the course of therapy. We assessed if the microbiota is able to return to its original profile after chemotherapeutic and antibiotic intervention with special interest in the abundance of *C. difficile*. We used a combination of molecular methods to compare abundance (qPCR) and diversity (PCR-DGGE) of bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV*, *Clostridium* cluster *XIVa* and *C. difficile* between groups and different time points of chemotherapy.

As mentioned above, we used feces as source of information. Fecal microbial communities are composed of autochthonous gut members and by transient bacteria. Even though the analysis of fecal samples may overestimate the actual composition of the GIT microbiota of humans we chose fecal samples to investigate the microbial composition of the intestinal microbiota because they are easy to collect, do not involve any ethical issues and reflect shifts in microbial population composition (Turroni *et al.*, 2008).

In this study we used type strains *Bacteroides thetaiotaomicron*^{T}, *Bifidobacterium longum* ssp. *longum*^{T} and *C. difficile* as well as the clones *CL16* and *CC34* for quantification of fecal microbiota. However, a mixture of different strains for qPCR standards would show a better image of the human microbiota. Therefore absolute amounts should be considered as semiquantitative.

The majority of previous studies on the effect of chemotherapy on human fecal microbiota used standard microbiological culture techniques (Nyhlen *et al.*, 2002; Stringer *et al.*, 2007). While other studies focused on the colonization with pathogenic bacteria (Schalk *et al.*, 2009; van Vliet *et al.*, 2009) among patients with cancer and chemotherapy-induced diarrhea (Stringer *et al.*, 2007; Abd El-Atti *et al.*, 2009), to our knowledge, we are the first to show changes in fecal bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV*, *Clostridium* cluster *XIVa* populations in addition to *C. difficile* analysis. However, the results of our *Bacteroides* analysis are not in agreement with the results of Nyhlèn et al., 2007 (Nyhlen *et al.*, 2002) who showed increased counts of *Bacteroides* spp. of samples obtained during chemotherapy, but with the microflora stable in most patients. Discrepancies in changes of the *Bacteroides* abundance might be due to differences in detection techniques. Nyhlèn et al., 2007 also reported significantly increased counts of yeast in patients, making it a focus for further research in immunocompromised patients.

Our results indicate significant effects of chemotherapy and antibiotic treatment on intestinal microbiota. Despite high individual variations, we show a significantly lower absolute bacterial load in feces of patients receiving chemotherapy in comparison to

healthy controls. These findings are in line with data from van Vliet et al, 2009, who reported 100-fold lower total bacterial numbers during chemotherapy treatment than in healthy controls. In addition to lower abundance of bacteria and determined bacterial subgroups, PCR-DGGE fingerprints indicate lower diversity of bacteria and *Clostridium* cluster *XIVa* in oncology patients. The abundance of fecal microbiota decreases after cycles of chemotherapy. After the end of chemotherapeutic administration the bacterial abundance recovers within a few days sometimes even showing a "rebound-effect". Relative numbers of *Clostridium* cluster *IV* and *XIV* show great alterations due to chemotherapeutical interventions, while the genera *Bacteroides* and bifidobacteria seem to be less affected. Interestingly, oncology patients harboured significantly more *Clostridium* cluster *IV* at higher diversity compared to healthy controls. This suggests that this class of bacteria is less susceptible to disruption by chemotherapeutic intervention. The incidence of *C. difficile* in subjects ON009 and ON011 is accompanied by a decrease of the genera bifidobacteria and *Clostridium* cluster *IV.* Further research is needed to elucidate if there is a causal relationship between growth of *C. difficile* and decreased abundance of bifidobacteria and *Clostridium* cluster *IV*.

Species richness was assessed using PCR-DGGE fingerprinting. Each lane of a PCR-DGGE gel represents a microbial fingerprint of a fecal sample; each band within a lane corresponds to one bacterial species, although different species may sometimes be represented by the same band (van Vliet *et al.*, 2009). It has also been observed that one bacterial strain may form several bands due to multipe 16S rRNA operons eg *Escherichia coli*^{T} (FIG. 3). The limitations of DGGE in microbial analysis have been previously described (Muyzer & Smalla, 1998). Nevertheless, substantial information about species composition can be obtained from very complex microbial communities such as the gut microbiota (Muyzer & Smalla, 1998).

The oncology patients assessed here suffered from a variety of malignancies and received different chemotherapy treatment regimes. Only two participants (ON001 and ON008) had never received any cancer therapy before, while all others had a

history of chemotherapeutic treatment. Therefore we could not hypothesize changes due to the beginning of the treatment. Most cancer patients also receive antibiotics. Microbial profiles were similar in individuals under chemotherapy regardless if they received antimicrobial treatment or not. The effects on the intestinal microbiota described here are thus unlikely to be due to antibiotics alone. Van Vliet et al. 2009 tested the effect of chemotherapy in vitro and showed a direct bacteriostatic effect of chemotherapeutics on bacterial growth.

Further research is needed to show whether changes in bacterial colonization play a role in the development and maintenance of mucosal barrier function, infection and inflammation (van Vliet *et al.*, 2009).

The use of prebiotics, probiotics and bacterial products, such as butyrate to prevent mucosal barrier injury and its complications could be a promising concept in restoring impaired functions or enhancing specific desirable functions of the microbiota (Zwielehner *et al.*, 2009). The use of pro- and prebiotics to affect the composition and metabolic activity of the fecal microbiota in times of cancer chemotherapy and immunosuppression might be part of future research.

In conclusion, chemotherapy treatment causes changes in fecal microbiota, which coincides with the development of *C. difficile* infection in some patients. These changes in microbiota may have systemic effects and may contribute to the development of chemotherapy-induced mucositis, influencing important beneficial functions of the microbial ecosystem.

Acknowledgements

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Domig for cultivation of type strains. The Hochschuljubiläumsfond of the Austrian National Bank funded this study.

FIGURES

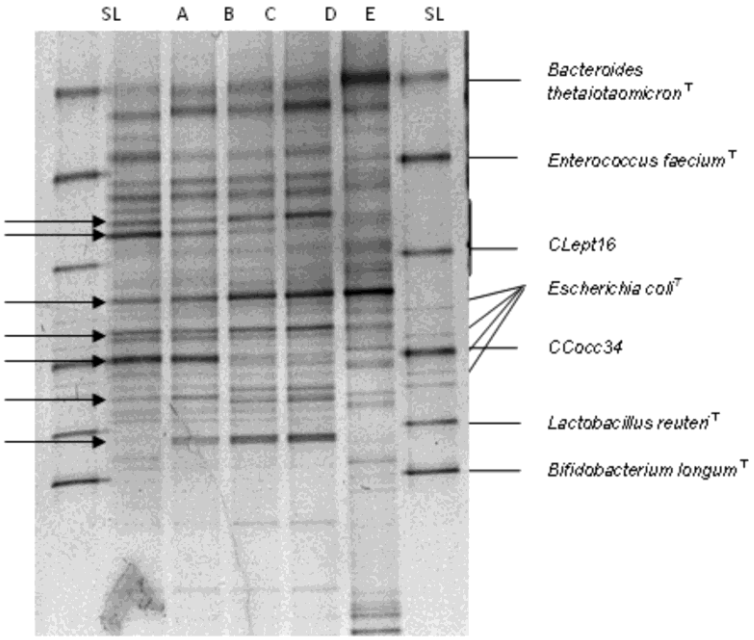


Figure 1: Changes of PCR-DGGE fingerprinting of 16S rRNA coding regions of dominant bacteria after chemotherapeutic treatment. Bands that become stronger or nearly disappear are indicated with arrows.

A, B, C and D, samples of ON001 after chemotherapy; E, healthy control; SL, standard lane.

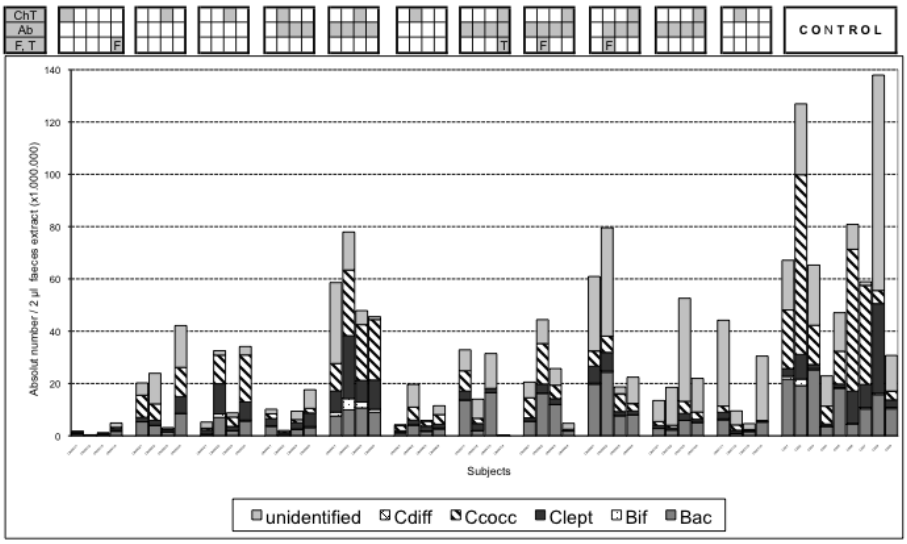


Figure 2: Impact of medical treatment (chemotherapy and antibiotics) on human fecal microbiota. Absolute numbers of *Bacteroides* (Bac), bifidobacteria (Bif), *Clostridium* cluster *IV* (Clept) and *XIVa* (Ccocc), *C. difficile* (Cdiff) and unidentified bacteria analyzed by TaqMan-qPCR.

ChT, Chemotherapy; Ab, Antibiotics; F, fever; T, blood stem cell transplantation; Subject ON001 received chemotherapy before first sampling. Subjects C001- C009 belong to the healthy age- and gender matched control group.

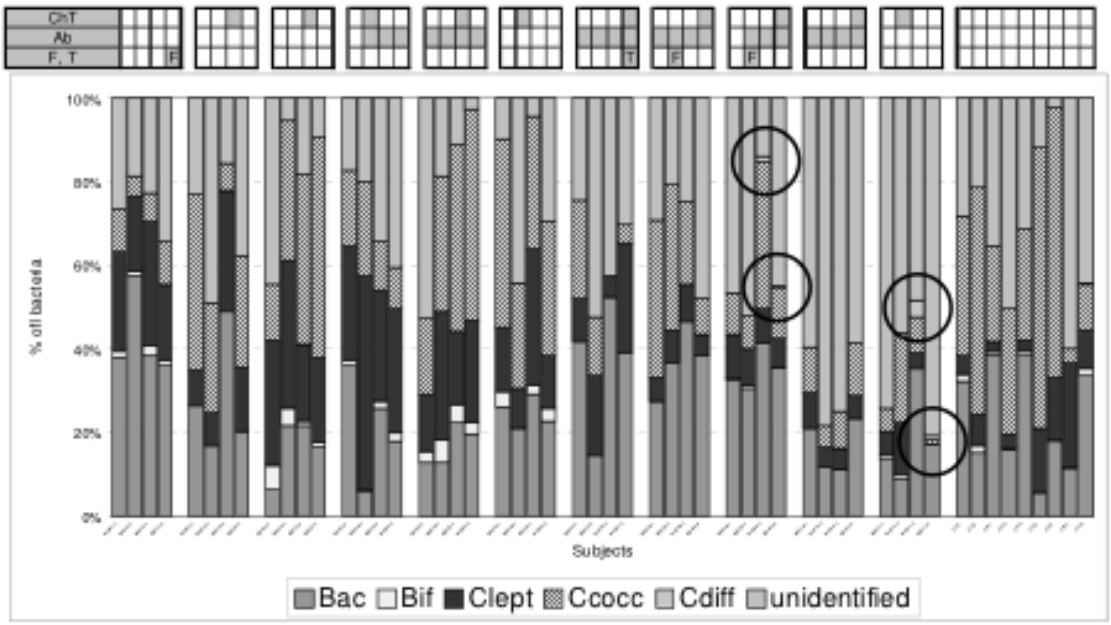


Figure 3: Percentage of *Bacteroides* (Bac), bifidobacteria (Bif), *Clostridium* cluster *IV* (Clept) and *XIVa* (Ccocc), *C. difficile* (Cdiff) and unidentified bacteria of analyzed bacteria quantified by TaqMan-qPCR. Red circle indicates *C. difficile.*

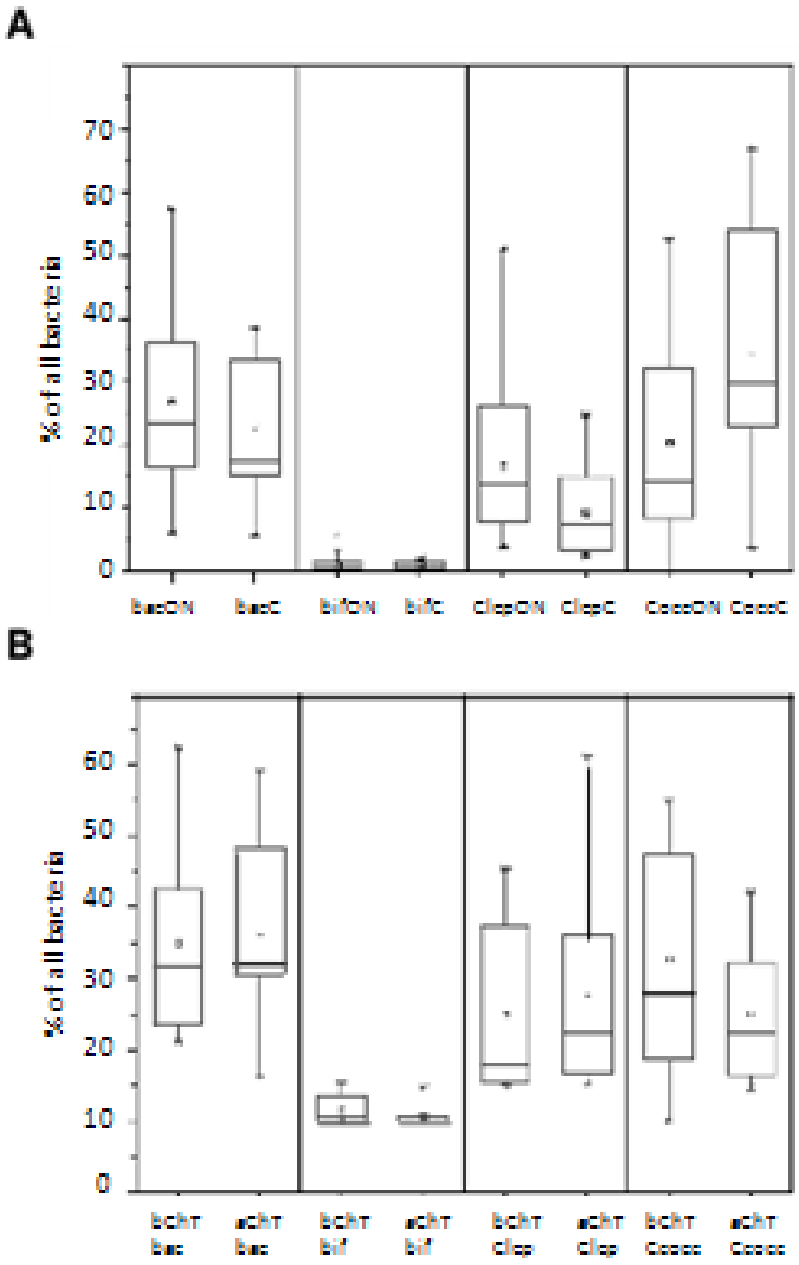


Figure 4. (A) Relative abundances of fecal bacteria in patients receiving chemotherapy ± antibiotics in comparison to healthy individuals. (B) Comparison of samples before and after chemotherapeutic cycles. Relative abundances of *Bacteroides* (bac), bifidobacteria (bif), C*lostridium* cluster *IV* (Clept) and cluster *XIVa* (Ccocc) are given relative to total bacteria.

ON, oncology patient; C, healthy control; bac, *Bacteroides;* bif, bifidobacteria; Clept, *Clostridium* cluster *IV*; Ccocc, *Clostridium* cluster *XIVa*

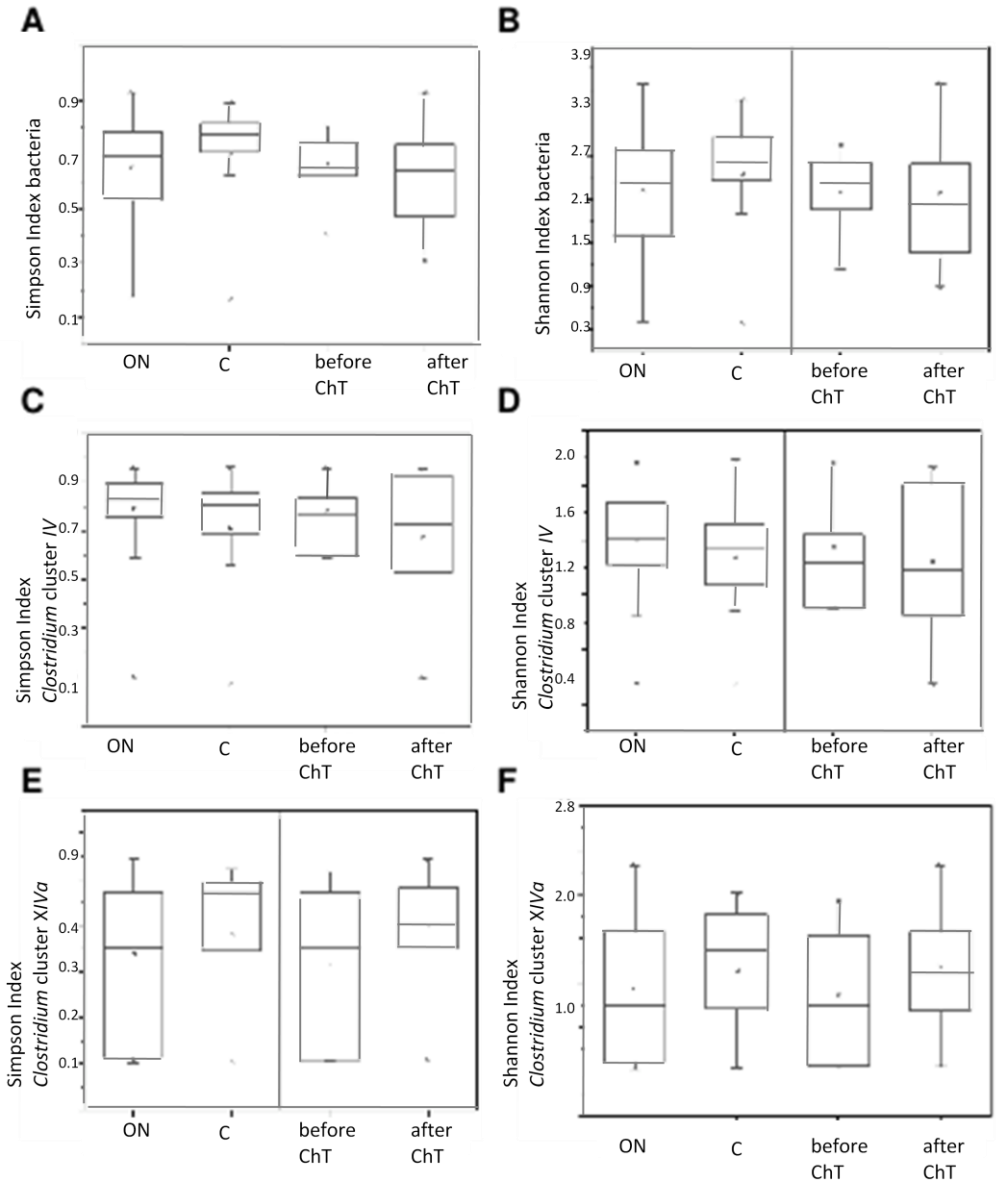
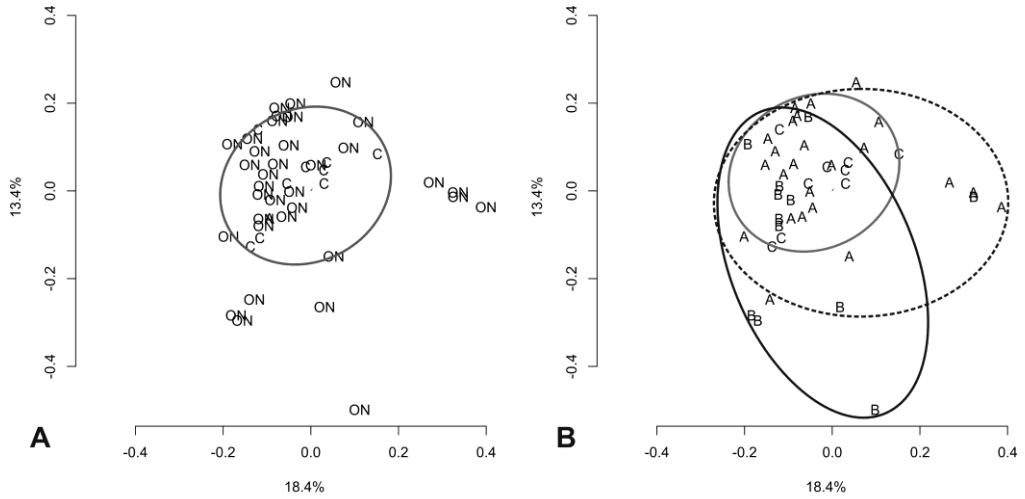


Figure 5: Simpson and Shannon diversity indices derived from PCR-DGGE bandpatterns of 16S rRNA coding regions of (A, B) bacteria, (C, D) *Clostridium* cluster *IV* and (E, F) *Clostridium* cluster *XIVa.*

ON, Oncology patients; C, healthy controls; ChT, chemotherapy



Fure 6: Principal component analysis (PCA) of *Clostridium* cluster *IV* DGGE fingerprints of 16S rRNA coding regions in fecal samples of ambulant oncology patient and healthy controls.

O, oncology pateints; C, healthy control; A, after chemotherapy; B, before chemotherapy.

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11 Summary

11.1 Aim

The aim of this Ph.D. thesis was to test if epidemiologically defined population groups such as vegetarians, omnivores, the young and the elderly differ in their microbiota composition. Furthermore, the influences of chemotherapy and antibiotic treatment on the GI microbiota should be monitored during the course of treatment.
Faecal samples of young healthy omnivores and vegetarians, institutionalized elderly and individuals undergoing immune-suppressive chemotherapy were analysed. Diet and lifestyle were assessed using an interviewer-conducted questionnaire.

The objective was to compare abundance and species richness of *Bacteria* as well as subgroups such as bifidobacteria, *Bacteroides, Clostridium leptum* cluster, *Clostridium coccoides* cluster in faecal samples of omnivores, vegetarians, elderly and individuals undergoing chemotherapy.

Molecular methods such as PCR-DGGE fingerprinting and qPCR as well as stable isotope probing were applied.
To allow meaningful interpretation of the highly diverse DGGE fingerprinting datasets, clustering based on Pearson correlation and principal components analysis based on covariance were applied. As a step towards a functional characterization, the butyryl-CoA CoA transferase gene was targeted in addition to the 16S rRNA gene used for phylogenetic identification. RNA-stable isotope probing was explored as a means of linking phylogenetic information with functional characteristics of gut microbial communities.

11.2 Introduction

The highly diverse microbiota of the human gastrointestinal tract has been associated with the pathogenesis of colorectal cancer [111], inflammatory bowel diseases [112, 113], obesity [27], metabolic syndrome [8] and atopic diseases [114]. The most predominant subpopulations in the human faecal microbiota are the *Clostridium leptum* subgroup, the *Clostridium coccoides subgroup* and *Bacteroides* [6, 115, 54]. Members of these populations contain short chain fatty acid (SCFA) producing fibrolytic bacteria [116, 117]. Another important subgroup of the human microbiota is that of the bifidobacteria. Stimulation of these bacteria has previously been shown after prebiotic intervention with inulin and fructo-oligosaccharides [92]. Bifidobacteria have been shown to be involved in prevention of atopic disease [122], and prevention of obesity and insulin resistance *via* enhanced barrier function of the gut epithelium [9]. SCFAs are derived from fermentation of non-digestible carbohydrates in the colon. The amount of SCFAs (primarily acetate, propionate and butyrate) produced in the colon depends on the site of fermentation, the diet and the composition of the residing microbiota, and can account for 5-15% of the total energy requirements for humans [173]. Due to rapid absorption and metabolism of SCFAs, their concentrations in the colon may differ. Acetate and propionate are metabolites for gluconeogenesis in the liver [15]. Butyrate is the major energy source for colonocytes and is known to regulate various processes in the human distal gut. Butyrate may have an effect on inflammation [158], oxidative stress [158], intestinal barrier function [159, 174, 175], visceral perception and rectal compliance [176] and may play a role in satiety [177, 178]. The formation of toxic products and lower availability of SCFAs in the distal colon were suggested to be involved in the pathogenesis of ulcerative colitis and cancer [179, 180, 181]. It has previously been described that the colon epithelium very efficiently removes butyrate from the faecal stream [183]. Thus, measuring butyrate levels in faeces might be a less suitable biomarker for intestinal health than the abundance of butyrate-producing bacteria or functional genes involved in butyrate production.

11.3 Results and Discussion

11.3.1 Vegetarian Microbiota

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 [123]. Different lifestyle factors and diets were shown to have a significant impact on the faecal microbiota [124, 109, 125]. Vegetarians had on average 12% more faecal bacteria than omnivores. Stephen et al showed in his study that a decreased transit time of stool is significant correlated to a higher bacterial cell mass [131]. The dietary questionnaire confirmed that vegetarians had a higher dietary fibre intake, which might have lead to a decreased transit time [132]. The higher abundance of bacteria in vegetarians may be attributed to that fact.

Furthermore, a tendency for higher proportions of *Bacteroides* and lower proportions of *Clostridium* cluster IV was found 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 [133, 134]. Nevertheless, members of *Clostridium leptum* subgroup have the same ability. One explanation could be that the particular dietary fibres enhance the development of *Bacteroides* more in the omnivore volunteers.

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 [109]. Therefore, we suppose that higher meat consumptions may increase the abundance of the *Clostridium leptum* subgroup in the gut microbiota. A higher prevalence of this subgroup was discussed to be associated with obesity and cancer [111, 125], where also increased diversity was observed. 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 *Ruminococcus* sp. were more prevalent in omnivores than in vegetarians. As this two subspecies are known as dietary fibre degraders we did not expect this finding. Two possible explanations for this observation may be the before discussed fact of a higher meat consumption or the very complex network of cross-feeding species. However, certainly further investigations are needed to clarify this aspect. The *Clostridium* cluster XIVa also tended to be more abundant in omnivores (19.01 ± 6.7%) than in vegetarians (14.52 ± 5.6%). The clostridial clusters IV and XIVa harbour many butyrate producing bacteria, e.g. *Faecalibacterium prausnitzii, Eubacterium* spp. and *Roseburia* spp.

Quantification of the butyryl-CoA CoA-transferase gene revealed a tendency towards higher levels of this important gene in vegetarians (106.89 ± 164.41ng/µl) than in omnivores (38.66 ± 35.04 ng/µl). Again, high individual differences were observed so that no significant differences could be found in young healthy omnivores and vegetarians. Butyryl-CoA CoA transferase gene abundance did not correlate with the overall abundance of bacteria.

In a melt curve analysis three groups of butyryl-CoA CoA transferase genes were attributed to species related to *E.hallii*/SS2/1, *Roseburia/E.rectale* spp. and *F.prausnitzii*. The abundance of these gene variants did not correlate with the abundance of the clostridial clusters IV and XIVa.

Vegetarian diet might have –in some individuals- favoured growth of *Roseburia/E.rectale* spp. that carry the butyryl-CoA CoA transferase gene, without causing an increase of the entire *Clostridium* cluster XIVa.

In other vegetarians, *Clostridium* cluster XIVa occurred at high abundance without a concomitant increase of the butyryl-CoA CoA transferase gene of *Roseburia*/*E.rectale* spp.

Omnivores tended to have higher abundances of *Clostridium* cluster IV and XIVa than vegetarians. The butyryl-CoA CoA transferase gene tended to be more abundant in

vegetarians. In conclusion, both vegetarian as well as omnivore diet have a similar potential to shape a healthy microbiota. The quantification of the butyryl-CoA CoA transferase gene is a valuable biomarker in addition to quantification of bacterial subgroups of the human GI microbiota.

11.3.2 Elderly microbiota

With ageing, decreases in beneficial organisms such as Lactobacilli and Bifidobacteria, amongst other anaerobes, and an increase in the number of facultative anaerobes [91] have been reported. Faeces from the institutionalized elderly had less total Bacterial abundance and lower total Bacterial diversity than that from the young subjects. In agreement with previous studies [13, 106, 95] ageing was associated with a loss of diversity of *Bifidobacteria*. 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. The *Clostridium cluster* XIVa was significantly more abundant in the young ($p=0.0018$) than in the elderly.

Furthermore, cluster analysis revealed that for all microbial groups analysed, the members of the GI microbiota in the elderly could be considered a subset of that present in the young.

Elderly had significantly less copies of the butyryl-CoA CoA-transferase gene than young omnivores. On an individual scale butyryl-CoA CoA transferase gene abundance did not correlate with overall bacterial 16S rRNA gene abundance.

The butyrate gene variant related to *Roseburia/E.rectale* spp. tended to be lower in the elderly than in the young. At the same time the *Clostridium* cluster XIVa was more abundant in the young. The abundance of the *Clostridium* cluster XIVa did not

correlate with the abundances of the butyrate gene variant related to *Roseburia/E.rectale* spp. on an individual scale.

Our results point out that elderly harbour less *Clostridium* cluster IV and XIVa in already low levels of overall bacterial. Together with lower abundance of the butyryl-CoA CoA transferase gene, our results indicate that the elderly microbiota might be characterized by low butyrate production capacity. Considering the important nutritive, anti-inflammatory and anticancerogenic potential of butyrate in the human colon, these findings point out that these microbiota specificities might contribute to the development of degenerative diseases [8] and anorexia in advanced age.

11.3.3 Butyrate concentration and assessment of bacterial butyrate removal

Faecal concentrations of butyrate ranged from below detection limit up to 0.094 µmol g^{-1} faeces (wt/w). The average value for omnivores was 0.023 µmol g^{-1} ± 0.027 µmol g^{-1} faeces, for elderly 0.007 µmol g^{-1} ± 0.011 µmol g^{-1} faeces and for vegetarians 0.008 µmol g^{-1} ± 0.008 µmol g^{-1} faeces. Butyrate concentrations found in the faeces of omnivores were highest among all individuals. In spite of high inter-individual differences these values were significantly different from butyrate in faeces of elderly ($p= 0.04$). As previously suggested, butyrate levels in faecal samples did not correlate with measured abundance of the butyryl-CoA CoA transferase gene. Discrepancies in gene abundance and faecal butyrate might be due to different ability of the gut epithelium to absorb butyrate, variable gene expression rate and enzyme activity and possibly also due to bacterial removal of butyrate from the faecal stream.

In order to assess if gut bacteria are capable of consuming butyrate, a RNA-stable isotope probing experiment was performed. In this experiment labelled butyrate was fed to an anaerobically incubated faecal sample. Butyrate consuming microorganisms were subsequently identified because they incorporated the heavy label in their RNA.

Some previously undescribed species of *Clostridiales* and *Erysipelotrichaceae* were identified. Nevertheless the results suggest that bacterial butyrate removal from the faecal stream is likely to be a minor process in the human gastro-intestinal tract.

11.3.4 Microbiota in individuals undergoing immune suppressive chemotherapy

Chemotherapy and antibiotics are used as life-prolonging measures for critically ill patients and to fight life-threatening infections but are associated with severe side effects such as mucositis, diarrhoea or constipation [144]. Chemotherapeutic and antibiotic treatment has a detrimental impact on the host microbial ecosystem, which is important for host mucosal protection [141] and thereby increases the risk of infection [137]. Overgrowth of species with potential pathogenicity such as toxigenic *C. difficile* and inflammatory complications are among the most common serious complications of chemotherapy and antibiotic treatment among patients with cancer [138, 137].

Despite high individual variations, a significantly lower absolute bacterial load was found in faeces of patients receiving chemotherapy in comparison to healthy controls. These findings are in line with data from van Vliet et al, 2009, who reported 100-fold lower total bacterial numbers during chemotherapy treatment than in healthy controls. In addition to lower abundance of bacteria and determined bacterial subgroups, PCR-DGGE fingerprints indicate lower diversity of bacteria and *Clostridium* cluster *XIVa* in oncology patients. The abundance of faecal microbiota decreased after cycles of chemotherapy. After the end of chemotherapeutic administration the bacterial abundance recovered within a few days sometimes even showing a "rebound-effect". Relative numbers of *Clostridium* cluster *IV* and *XIV* show great alterations due to chemotherapeutical interventions, while the genera *Bacteroides* and bifidobacteria seem to be less affected. Interestingly, oncology patients harboured significantly more *Clostridium* cluster *IV* at higher diversity compared to

healthy controls. This suggests that this class of bacteria is less susceptible to disruption by chemotherapeutic intervention. The incidence of *C. difficile* in subjects ON009 and ON011 was accompanied by a decrease of the *Bifidobacteria* and *Clostridium* cluster *IV.* Further research is needed to elucidate if there is a causal relationship between growth of *C. difficile* and decreased abundance of *Bifidobacteria* and *Clostridium* cluster *IV*. In conclusion, chemotherapy treatment causes changes in faecal microbiota, which coincide with the development of *C. difficile* infection in some patients. These changes in microbiota may have systemic effects and may contribute to the development of chemotherapy-induced mucositis, influencing important beneficial functions of the microbial ecosystem.

11.4 Conclusion

The chosen approach combining quantitative PCR and qualitative PCR-DGGE fingerprinting showed that the human microbiota is highly variable between individuals. In spite of this enormous variability, some significant differences between elderly and young could be shown. Young healthy vegetarians and omnivores tended to have characteristic microbiota as well, but the differences between these two epidemiologically defined populations were smaller than the interindividual differences. Interestingly, the *Clostridium leptum* cluster which harbors fiber-degrading and butyrate-producing bacteria, was more diverse in individuals undergoing immune-suppressive chemotherapy and more abundant in young healthy omnivores. As a first step towards a characterization of microbiota functions, quantification of an important functional gene involved in butyrate production was performed. RNA-stable isotope probing was explored as a means to link phylogeny with function. Further research is needed to elucidate causal relationships between human behavior and microbiota composition and function.

12 Zusammenfassung

12.1 Zielsetzung

Die Fragestellung der vorliegenden Doktoratsarbeit war, ob epidemiologisch definierte Bevoelkerungsgruppen wie VegetarierInnen, MischkoestlerInnen, alte und junge Menschen sich hinsichtlich ihrer Mikrobiota-Zusammensetzung unterscheiden. Weiters war es ein Ziel die Einfluesse von Chemotherapie und Antibiotika auf die GI-Mikrobiota im Vergleich zu gesunden Kontrollen zu charakterisieren. Stuhlproben von jungen Omnivoren, Vegetarierinnen, AltersheimbewohnerInnen und Individuen, die immunsuppressive Chemotherapie erhalten, wurden untersucht. Ernaehrungsgewohnheiten und Lebensstilfaktoren wurden in Interviews anhand eines Fragebogens erfasst.

Moegliche Unterschiede sollten anhand der Abundanz und Fuelle an Spezies (richness) der dominierenden Bakterien und Bifidobakterien, *Clostridium leptum* cluster, *clostridium coccoides* cluster und *Bacteroides* beschrieben werden.

Ausschliesslich molekulare Methoden, naemlich PCR-DGGE fingerprinting, quantitative PCR und stable isotope probing wurden angewandt. Um sinnvolle Interpretation der hoch diversen DGGE fingerprinting Daten zu erlauben, wurde Clustering basierend auf Pearson Korrelation und Hauptkomponentenanalyse basierend auf Kovarianz angewandt. Als ein Schritt in Richtung funktioneller Charakterisierung wurde das Butyryl-CoA CoA Transferase Gen zusaetzlich zur Quantifizierung des 16S rRNA Gens, anhand dessen phylogenetische Identifizierung vorgenommen wurde, durchgefuehrt. RNA-stable isotope probing wurde als vielversprechende Methode identifiziert, um phylogenetische Information mit funktionellen Charakteristika von mikrobiellen Gemeinschaften des Darms zu verbinden.

12.2 Einleitung

Die hoch diverse Mikrobiota des menschlichen gastro-intestinalen Trakts wurde mit der Pathogenese von Kolorektalkrebs [111], chronisch entzuendlichen Darmerkrankungen [112, 113], Adipositas [27], metabolischem Syndrom [8] und atopischen Erkrankungen [114] in Zusammenhang gebracht. Die dominanten Subpopulationen der menschlichen Darmbakterien sind dem *Clostridium leptum* Cluster, dem *Clostridium coccoides* cluster und den *Bacteroides* zuzuordnen [6, 115, 54]. Mitglieder dieser Populationen beinhalten fibrolytische Bakterien, die kurzkettige Fettsaeuren (SCFAs) produzieren [116, 117]. Eine weitere wichtige Untergruppe der menschlichen Mikrobiota ist die der Bifidobakterien. Stimulation dieser Bakterien wurde in der Vergangenheit durch prebiotische Intervention mit Inulin und Fructooligosacchariden beschrieben [92]. Bifidobakterien wurden mit der Praevention von atopischen Erkrankungen [122] und der Praevention von Adipositas und Insulinresistenz durch Verbesserung der Barrierefunktion des Darmepithels [9] assoziiert. SCFAs sind Derivate aus der Fermentation von unverdaulichen Kohlenhydraten im Colon. Welche Menge an SCFAs (hauptsaechlich Acetat, Propionat udn Butyrat) gebildet wird, haengt von dem Fermentationsort, der Ernaerung und der Zusammensetzung der Mikrobiota ab. SCFAs koennen 5-15% des menschlichen Energiebedarfs decken [173]. Die Konzentration von SCFAs im Colon koennen unterschiedlich hoch sein, weil sie rasch absorbiert und metabolisiert werden. Acetat und Propionat sind Metaboliten fuer die Gluconeogenese in der Leber [15]. Butyrat ist die Hauptenergiequelle fuer Colonocyten und reguliert diverse Prozesse im distalen Darm. Butyrat hat moeglicherweise einen Einfluss auf Entzuendungen [158], oxidativen Stess [158], intestinale Barrierefunktion [159, 174, 175], viscerale Wahrnehmung und rektale Compliance [176] und koennte im Zusammenhang mit Hunger und Saettigung eine Rolle spielen [177, 178]. Es wurde vorgeschlagen dass die Bildung toxischer Produkte und geringere Verfuegbarkeit von SCFAs im distalen Colon in die Pathogenese von Ulcerativer Colitis und Krebs [179, 180, 181] involviert sind. Es wurde beschrieben, dass das Colonepithel sehr effizient Butyrat aus dem faekalen

Strom entfernt [183]. Daher scheint Butyratbestimmung in Stuhl als Darmgesundheits-Biomarker weniger geeignet als die Abundanz Butyrat-produzierender Bakteren oder funktioneller Gene, deren Produkte in der Butyratproduktion involviert sind.

12.3 Resultate und Diskussion

12.3.1 Mikrobiota in VegetarierInnen

Vegetarismus wurde mit geringerem Krankheitsrisiko fuer Herzerkrankungen und verschiedenen Krebsarten assoziiert. Weiters wurden niedrigerer BMI und insgesamt niedrigere Mortalitaetsrate in Vegetariern beschrieben [123]. Verschiedene Lebenstile und Ernaehrungsformen [124, 125, 109] wurden als signifikante Einflussfaktoren auf die faekale Mikrobiota beschrieben. VegetarierInnen hatten durchschnittlich 12% mehr faekale Bakterien als Omnivoren. Stphen et al. zeigten, dass eine verringerte Stuhl- Transitzeit signifikant mit einer hoeheren bakteriellen Zellmasse korreliert [131]. Die Ernaehrungserhebung zeigte, dass Vegetarier eine hoehere Faserstoffaufnahme hatten, was zu einer verringerten Darmpassagezeit gefuert haben koennte [132]. Die hoehere Abundanz von Bakterien koennte darauf zurueckzufuehren sein.

Weiters, wurde in VegetarierInnen im Vergleich zu Omnivoren eine Tendenz fuer einen hoeheren *Bacteroides* Anteile und einen niedrigeren Anteil von *Clostridium* cluster IV gefunden. *Bacteroides* koennen ein breites Spektrum an Kohlenstoffquellen verwerten, sie sind hauptverantwortlich an der Polisaccharidverdauung im Dickdarm beteiligt [133, 134]. Bakterien des *Clostridium leptum* cluster haben die gleichen Faehigkeiten. Moeglicherweise foerdern bestimmte Faserstoffe in der omnivoren Ernaehrung die *Bacteroides* mehr als unverdauliche Polysaccharide der vegetarischen Ernaehrung.

Mueller et al. fanden die hoechsten Anteile von *Clostridium* cluster IV in ihrer schwedischen Studienpopulation, deren Ernaehrung von hohem Fleisch- und

Fischkonsum charakterisiert war [109]. Daher liegt die Vermutung nahe, dass Fleischkonsum die Mikrobiota-Populationen des *Clostridium leptum* clusters besonders foerdert. Eine hoehere Prevalenz dieser Subgruppe wurde auch im Zusammenhang mit Adipositas und Krebs [111, 125] diskutiert, wo auch eine erhoehte Diversitaet beobachtet wurde. PCR-DGGE fingerprinting der dominanten Bakterien und *Clostridium* cluster IV resultierte in Gruppierung von VegetarierInnen und Omnivoren. Clustering der *Clostridium leptum* Subgruppe anhand der DGGE fingerprints koennte darauf zurueckzufuehren sein, dass zwei Sequenzen, die als *Faecalibacterium* sp. und *Ruminococcus* sp. identifiziert wurden, in Omniovren haeufiger vorkamen als in VegetarierInnen in der hier vorliegenden Arbeit. Da diese zwei Spezies als Degradierer von Faserstoffen bekannt sind, war diese Beobachtung unerwartet. Zwei moegliche Erklaerungen fuer diese Beobachtung koennte der oben diskutierte Fleischkonsum sein, zum anderen koennte dieser Unterschied auch auf die komplexen Nahrungsstrategie des “cross-feeding“ im Mikrobiota zurueckzufuehren sein. Fuer eine eindeutige Interpretation dieses Aspekts sind zukuenftige Forschungen vonnoeten. Der *Clostridium coccoides* cluster (XIVa) zeigte die Tendenz in Omnivoren (19.01 ± 6.7%) haeufiger vorzukommen als in VegetarierInnen (14.52 ± 5.6%). Sowohl der *Clostridium* cluster IV als auch der cluster XIVa beinhalten viele Butyrat-produzierende Spezies, z.B.: *Faecalibacterium prausnitzii, Eubacterium* spp. und *Roseburia* spp.

Quantifizierung des Butyryl-CoA CoA transferase Gens liess eine Tendenz zu hoeheren Levels dieses Gens in Vegetariern (106.89 ± 164.41ng/µl) als in Omivoren (38.66 ± 35.04 ng/µl) erkennen. Wieder wurden grosse indivudelle Unterschiede beobachtet, weswegen keine signifikanten Unterschiede zwischen jungen, gesunden Omnivoren und VegetarierInnen gefunden werden konnten. Die Abundanz des Butyryl-CoA CoA transferase Gens korrelierte nicht mit der Gesamthaeufigkeit der Bakterien.

In einer Schmelzkurvenanalyse wurden drei Varianten des Butyryl-CoA coA transferase Gens identifiziert, die Spezies um *E.hallii* / SS2/1, *Roseburia*/*E.rectale* spp. und *F.prausnitzii* zugeordnet wurden. Die Abundanz dieser drei Genvarianten korrelierten

nicht mit den Abundanzen der beiden clostridialen cluster IV und XIVa. Vegetarische Ernaehrung hat wohl in manchen Individuen das Aufkommen von *Roseburia*/*E.rectale* spp., die diese Variante des Butyratgens tragen, gefoerdert, ohne den *Clostridium* cluster XIVa insgesamt in seiner Abundanz zu erhoehen. In anderen VegetarierInnen war der cluster XIVa sehr abundant, ohne aber mit einer hohen Abundanz der *Roseburia*/*E.rectale* Butyratgenvariante einherzugehen.

Omnivoren tendierten im Vergleich zu VegetarierInnen zu einer hoeheren Abundanz der clostridialen Cluster IV und XIVa. Das Butyryl-CoA coA transferase Gen war jedoch abundanter in VegetarierInnen. Zusammenfassend laesst sich schliessen, dass sowohl die omnivore als auch die vegetarische Ernaehrung ein aehnliches Potential haben, eine gesunde Mikrobiota zu formen. Die Quantifizierung des Butyryl-CoA coA transferase Gens kann zusammen mit der Quantifizierung bakterieller Subpopulationen der menschlichen Mikrobiota als nuetzlicher Biomarker dienen.

12.3.2 Die Zusammensetzung der Mikrobiota betagter Menschen

Es wurde beschrieben, dass mit hoeherem Alter eine Abnahme von Anaerobiern wie Laktobazillen und Bifidobakterien und eine Zunahme der fakultativ anaeroben Bakterien einhergeht [91]. Faeces von betagten Bewohnern eines Pflegeheims hatten insgesamt weniger Bakterien und geringere Diversitaet als junge MischkoestlerInnen. In Uebereinstimmung mit frueheren Studien [13, 106, 95] war auch hier das hohe Alter mit einer Abnahme der Bifidobakterien-Diversitaet assoziiert. *Bacteroides* nahmen einen hoehern Anteil an der Mikrobiota ein, jedoch bei tendenziell niedrigerer Diversitaet als in Jungen. Die relative Abundanz der Bifidobakterien und *Clostridium* cluster IV war in Jungen signifikant hoeher als in Betagten. Der *Clostridium leptum* cluster war ausserdem diverser in den jungen als in den betagten StudienteilnehmerInnen. Der *Clostridium* cluster XIVa war signifikant (p=0.0018) haeufiger in Jungen als in Betagten.

Weiters zeigte die Clusteranalyse der DGGE fingerprints, dass die Mikrobiota von aelteren Menschen als ein Subset der jungen Mikrobiota angesehen werden kann.

Betagte hatten signifikant weniger Kopien des Butyryl-CoA coA transferase Gens as Junge. Auf individualler Ebene korrelierte die Butyryl-CoA coA transferase Gen Abundanz nicht mit der Abundanz des bakteriellen 16S rRNA Gens.

Die Butyrat- Genvariante der *Roseburia/E.rectale* spp. war tendentiell niedriger in Betagten als in Jungen. Damit uebereinstimmend, nahm der *Clostridium* cluster XIVa in Jungen einen hoeheren Anteil der Mikrobiota ein. Die Abundanz des clusters XIVa korrelierte nicht mit der Abundanz des *Roseburia/E.rectale* spp. Butyryl-CoA coA transferase Gens.

Unsere Resultate weisen darauf hin, dass die Mikrobiota betagter Menschen weniger *Clostridium* cluster IV und XIVa in insgesamt weniger Bakterien aufweist. Zusammen mit niedrigen Levels des Butyryl-CoA CoA transferase Gens kann geschlossen werden, dass die Mikrobiota in hohem Alter eine geringere Butyratproduktionskapazitaet hat. Unter Beruecksichtigung der wichtigen nutritiven, anti-inflammatorischen und antikanzerogenen Eigenschaften des Butyrat ist es durchaus vorstellbar dass diese Mikrobiota Eigenschaften zur Entwicklung degenerativer Erkrankungen [8] und Anorexie im hohen Alter beitraegt.

12.3.3 Butyratkonzentrationen und der Beitrag bakteriellen Butyratverbrauchs

Faekale Butyrat-Konzentrationen reichten von unter dem Detektionslimit bis zu 0.094 µmol g^{-1} faeces (wt/w). Durchschnittswerte waren fuer Omnivoren 0,023 µmol g^{-1} ± 0,027 µmol g^{-1} faeces, fuer VegetarierInnen 0.008 µmol g^{-1} ± 0.008 µmol g^{-1} und fuer Betagte 0.007 µmol g^{-1} ± 0.011 µmol g^{-1} faeces. Die Butyratkonzentrationen waren in Omnivoren am hoechsten unter allen StudienteilnehmerInnen. Trotz hoher individueller Unterschiede waren sie signifikant hoeher als in Betagten (p=0.04). Wie

schon frueher beschrieben, korrelierten die gemessenen Butyratkonzentrationen nicht mit der Abundanz des Butyryl-CoA CoA transferase Gens. Diese Diskrepanz koennte aufrund unterschiedlicher Absorptionsrate des Epithels, variabler Genexpression und Enzymaktivitaet und moeglicherweise auch durch bakteriellen Verbrauch des gebildeten Butyrats zustande gekommen sein.

Um die Moeglichkeit zu pruefen, dass Darmbakterien Butyrat verbrauchen koennen, wurde ein RNA-stable isotope proing (RNA-SIP) Experiment durchgefuehrt. In diesem Experiment wurde eine Stuhlprobe unter anaeroben Bedingungen mit ^{13}C-Butyrat inkubiert. Butyrat konsumierende Bakterien konnten daraufhin identifiziert werden, weil sie den schweren Kohlenstoff in ihre RNA inkorporiert hatten. Einige zuvor nicht beschriebene Spezies der *Clostridiales* und *Erysipelotrichaceae* wurden identifiziert. Die Resultate weisen darauf hin, dass bakterieller Verbrauch der Buttersaeure ein unwesentlicher Prozess im menschlichen gastro-intestinalen Trakt ist.

12.3.4 Mikrobiota in Individuen unter immunsuppressiver Chemotherapie

Chemotherapie und die Gabe von Antibiotika sind lebensverlaengernde Massnahmen fuer kritisch kranke PatientInnen und helfen, lebensbedrohliche Infektionen abzuwehren. Sie gehen of mit schweren Nebenwirkungen wie Mukositis, Diarrhoe oder Obstipation [144] einher. Chemotherapie und Antibiotika haben einen nachteiligen Einfluss auf das mikrobielle Oekosystem, das fuer den mukosalen Schutz des Menschen [141] wichtig ist, und erhoeht so das Infektionsrisiko [137]. Ueberbordendes Wachstum von Spezies mit potentieller Pathogenitaet wie toxigene *C.difficile* und entzuendliche Komplikationen sind mit die schwersten Komplikationen der Chemotherapie und Antibiotikagabe bei KrebspatientInnen [138, 137].

Trotz hoher indivieller Variationen wurden signifikant weniger Bakterien in Chemotherapie-PatientInnen gefunen als in gesunden Kontrollen. Diese Beobachtung bestaetigt eine fruehere Arbeit von van Vliet et al. 2009, die eine 100-fache Reduktion

der Darmbakterien waehrend Chemotherapie beschreibt. Zusaetzlich zu Verringerung der Bakterien insgesamt ergab PCR-DGGE fingerprinting geringere Diversitaet der Bakterien und *Clostridium* cluster XIVa in KrebspatientInnen. Die Abundanz der faekalen Mikrobiota nahm nach Chemotherapie-Zyklen ab. Nach dem Ende der Chemotherapiegabe erholte sich die Mikrobiota innerhalb weniger Tage und zeigt manchmal sogar einen Aufschwung ueber Anfangsniveau hinaus. Relative Haeufigkeit der *Clostridium* cluster IV und XIVa schwankten stark infolge Chemotherapie, wogegen *Bacteroides* und Bifidobakterien weniger stark betroffen waren. Interessanterweise wiesen PatientInnen hoehere relative Abundanz des *Clostridium* cluster IV als gesunde Kontrollen auf, sogar bei hoeherer Diversitaet. Dies legt den Schluss nahe, dass diese Gruppe von Bakterien weniger anfaellig fuer Chemotherapie ist. Die Inzidenz von *C.difficile* in PatientIn ON009 und ON011 ging mit einer Abnahme von Bifidobakterien und *Clostridium* cluster IV einher. Weitere Forschung ist noetig um zu klaeren, ob es zwischen dem Aufkommen von *C.difficile* und verringerter Abundanz von Bifidobakterien und *Clostridium* cluster IV einen kausalen Zusammenhang gibt.

Zusammenfassend ist festzuhalten, dass Chemotherapie die faekale Mikrobiota veraendert, und diese Veraenderungen mit dem Aufkommen von *C.difficile* in manchen PatientInnen einhergehen. Diese Veraenderungen koennten systemische Effekte haben und moeglicherweise zu Chemotherapie-induzierter Mukositis beitragen indem sie die nutzbringenden Funktionen der GI Mikrobiota beeinflussen.

12.4 Schlussbetrachtung

Die gewaehlte Herangehensweise mittels quantiative PCR und qualitativem PCR-DGGE fingerprinting zeigte das die menschliche Mikrobiota individuell sehr variabel ist. Trotz dieser enormen Variabilitaet konnten einige signifikante Unterschiede zwischen betagten und jungen Menschen gezeigt werden. Junge gesunde VegetarierInnen und Omnivoren hatten tendentiell charakteristische Mikrobiota-Zusammensetzungen, aber die Unterschiede zwischen den einzelnen Individuen waren groesser als die

Unterschiede zwischen diesen beiden epidemiologisch definierten Gruppen. Interessanterweise war der *Clostridium leptum* cluster, der Faserdegradierer und Butyratproduzenten beherbergt, in Chemotherapie-PatientInnen diverser als in gesunden Kontrollen und relativ haeufiver in Omnivoren als in VegetarierInnen. Als ein erster Schritt in Richtung Charakterisierung der Mikrobiota Funktionen, wurde ein fuer die Butyratproduktion wichtiges funktionelles Gen quantifiziert. RNA-stable isotope probing wurde als ein Werkzeug identifiziert, mit dem phylogenetische Information mit Funktion verlinkt werden kann. Weitere Forschungsarbeit ist von Noeten, um kausale Zusammenhaenge zwischen menschlichem Verhalten und der Zusammensetzung und Funktion der Mikrobiota zu beleuchten.

13 CURRICULUM VITAE

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EDUCATION

Nov 2006 - Apr2010	Ph.D. thesis **"Molecular analysis of fecal microbiota with PCR-DGGE fingerprinting and qPCR."** under supervision of Dr. A. Haslberger. Department of life sciences, University of Vienna, Austria.
May 2005 – Oct 2006	Diploma thesis "**Identification of microbial communities on lettuce"** under supervision of Dr. A. Haslberger.
Oct 1999 – Oct 2006	Diploma of Nutritional Science (*Mag.rer.nat.* – Equivalent of Masters Degree) Department of Life Sciences, University of Vienna, Austria.

PRACTICAL EXPERIENCE

Feb 2010 – Mar 2010	Lecturer, University of Vienna, Austria **"Quantification of genetically modified material in food"**

Dec 2008 - Mar 2009	Practicum student with Dr. Mike Manefield at the University of New South Wales, Australia. Applying RNA-stable isotope probing to identify butyrate-assimilating bacteria of the human GI-tract.
2008	Co-lecturer at the University of Vienna, Austria. **"Biotechnology and gene technology in food production"** (#330012, 3 ECTS credits)
2007-2010	Lecturer at the University of Vienna, Austria. Laboratory course for master students (#330052, 4.5 ECTS credits)
2006-2010	Honours lecturer and co-supervisor in the working group of Dr. Alexander Haslberger, University of Vienna, Austria. Successful preparation of research proposals aiming at the molecular analysis of human GI microbiota of individuals of different age, diet and health status such as, immune-compromised individuals.
2006-2010	PhD Thesis. PCR-DGGE fingerprinting & principal components analysis, quantitative PCR analysing human GI microbiota.
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Aug 2006	Ludwig Boltzmann Institute for Leukaemia research: Isolation of bacterial DNS from blood samples, PCR, DGGE and cloning.

Sept 2005	OfI- Österreichisches Institut für Forschung und Innovation; Vienna, Austria. Chemical food quality testing.
Aug 2003	BfR- Bundesinstitut für Risikobewertung; Berlin. Risk assessment of asthaxanthine dietary supplements.

AWARDS

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