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Changes in human faecal microbiota due to chemotherapy analyzed by TaqMan-PCR and PCR-DGGE

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3 OBJECTIVES

The human large intestine is colonized by a dense and complex community composed largely of anaerobic bacteria [FLINT et al., 2007]. This large and diverse microbiota is recognized as contributing to gut maturation, host nutrition and pathogen resistance. Moreover, microbes have been shown to regulate intestinal epithelial proliferation, host energy metabolism and inflammatory immune responses [DETHLEFSEN et al., 2006]. Additionally, the main functions of the microbiota include the fermentation of non-digestible dietary residue and endogenous mucus, salvage of energy as short-chain fatty acids (SCFAs), production of vitamin K and absorption of ions [GUARNER et al., 2003].

As mentioned above, the human gut microbiota plays several significant roles in the metabolism of nutrients. On the other hand, the composition of gut microbiota can be modulated by host, environment, bacterial factors and diet may exert a major effect on the composition and activity of the gut microbiota. Strong evidence has emerged of substantial modifications during illness (chronic and degenerative diseases including inflammatory bowel disease, Crohn's disease, colon cancer and rheumatoid arthritis) or exposure to threatening experiences, such as antibiotics and chemotherapy [THOMPSON-CHAGOYAN et al., 2007]. Evidence support the potential role of the gut microbiota in the development of obesity and explore the role that modifying the gut microbiota may play in its future treatment, as obesity is a major health problem because of its serious health consequences including type 2 diabetes and cardiovascular diseases [DIBAISE et al., 2008].

Diet-derived substrate, particularly undigested fibre and starch reaching the large intestine, have major effects upon bacterial community structure and metabolism in the colon. SCFAs formed by microbial fermentation have an important effect on colonic health. Butyrate in particular provides a potentially important link between diet and colonic health [BARCENILLA et al., 2000].

With ageing, a decrease in beneficial organisms such as Lactobacilli and Bifidobacteria and an increase in the number of facultative anaerobes have been reported. This, along with a general reduction in species diversity, changes to diet and altered digestive physiologies such as intestinal transit time, may result in increased gastrointestinal discomfort and a greater susceptibility to disease [ZWIELEHNER et al., 2009].

Even if chemotherapy and antibiotics are used for combating life-threatening diseases, treatment with antibiotics and chemotherapy may result in shifts of the human microbiota associated with side-effects like diarrhoea, constipation and malnutrition. The altered balance of the microbiota potentially leads to *Clostridium difficile* infections, a common colonizer of the human gut whose growth is held in check by the normal biota but which overgrows the biota upon antibiotic use and chemotherapeutic treatment. Because of these changes, absorption and other intestinal functions involving the microbiota are altered [CROSWELL et al., 2009, GIBSON et al., 2006, SCHALK et al., 2009].

Altogether, the microbiota seems to be one of the most complex ecosystems with a multitude of capacities as interacting with diet and the human host, moving the focus of this diploma thesis on the human microbiota. In particular, this thesis should give an overview of the topics mentioned above: microbiota and butyrate, the ageing microbiota and the microbiota in times of chemotherapy and antibiotic treatment.

The manuscripts titled “Changes in human faecal microbiota due to chemotherapy analyzed by TaqMan-PCR and PCR-DGGE fingerprinting” by Lassl et al., “Consequences of diet and ageing for individual SCFAs and faecal microbiota” by Hippe et al. and the already published article “Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in faecal population sizes and diversity of *Bacteroides*, Bifidobacteria and *Clostridium* cluster IV in industrialized elderly” by Zwielehner et al. can be found in the appendix of this thesis. The author of this thesis participated in writing and working on the

manuscript by Hippe *et al.* and the published research article by Zwielehner *et al.*, but the main focus of this diploma thesis is on analyzing the microbiota during chemotherapy and antibiotic treatment.

The purpose of this thesis was to analyze the course of faecal microbiota of patients receiving cancer chemotherapy $\pm$ antibiotics in comparison to healthy control individuals. The focus was on abundance and diversity of dominant bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster IV, *Clostridium* cluster XIVa and *Clostridium difficile* using culture-independent methods. Changes in populations were analyzed for the ability to return to its original composition (resilience) after chemotherapy and the emergence of *Clostridium difficile*.

4 INTRODUCTION

4.1 MICROBIOTA AND HOST

The adult human intestine is home to an almost inconceivable number of microorganisms [BACKHED *et al.*, 2005]. The microorganisms that live inside and on humans (known as the microbiota) are estimated to outnumber human somatic and germ cells by a factor of ten. Together, the genomes of these microbial symbionts (defined as the microbiome) provide traits that humans did not need to evolve on their own [TURNBAUGH *et al.*, 2007]. Therefore, our gut microbiota can be pictured as a microbial organ placed within a host organ [BACKHED *et al.*, 2005].

The gut microbiome encodes metabolic capacities that remain largely unexplored but include (1) the degradation of otherwise indigestible components of our diet [TURNBAUGH *et al.*, 2009], (2) protection against epithelial cell injury, (3) regulation of host fat storage [ECKBURG *et al.*, 2005], (4) vitamin synthesis and (5) generation of short-chain fatty acids [TLASKALOVA-HOGENOVA *et al.*, 2004]. Moreover, a healthy microbiota plays significant roles in the immunopotential and prevention of colonization by

pathogens in the gastrointestinal tract and hence is involved in maintaining human health [DE LA COCHETIERE et al., 2005].

We know that the composition of the GI microbiota is highly distinct between individuals and many factors, such as diet, host genotype and microbial interactions seem to be able to explain the diversity and the individuality of these communities [DETHLEFSEN et al., 2006]. Despite this variability, there appear to be 'core species' present in a majority of humans which are quite resilient to external influences and 'passengers' or transients, sometimes in great numbers, sometimes below detection limits [ZWIELEHNER et al., 2009].

Our knowledge of the composition of the adult gut microbiota mainly stems from culture-based studies [BACKHED et al., 2005], but due to insensitivity of cultivation, in the last decades investigators have begun to explore this ecosystem using molecular fingerprinting methods and sequence analysis of cloned 16S small-subunit ribosomal RNA (16S rRNA) genes [ECKBURG et al., 2005].

4.2 MICROBIOTA IN HEALTH AND DISEASE

The composition of gut microbiota can be modulated by host, environment, and bacterial factors and strong evidence has emerged of substantial modifications during illness or exposure to threatening experiences and medical treatment. Alterations in gut microbiota and their functions have been widely associated with many chronic and degenerative diseases including inflammatory bowel disease, colon cancer and rheumatoid arthritis. [THOMPSON-CHAGOYAN et al., 2007].

Crohn's disease, ulcerative colitis, and pouchitis are the result of continuous microbial antigenic stimulation of pathogenic immune responses as a consequence of host genetic defects in mucosal barrier function, innate bacterial killing, or immunoregulation. Altered microbial composition and function in inflammatory bowel disease result in increased immune stimulation, epithelial dysfunction, or enhanced mucosal permeability [SARTOR, 2008].

Correlations between the gastrointestinal microbiota and diseases like allergenicity [SHREINER et al., 2008], autoimmune diseases [TLASKALOVA-HOGENOVA et al., 2004] and individual dispositions such as obesity [ZHANG et al., 2009] have been discussed.

Under normal conditions, the gut microbial ecosystem is quite stable and humans are protected against infections by their anaerobic intestinal microorganisms, but can be negatively affected by external factors and these include antibiotic treatments [IAPICHINO et al., 2008] and chemotherapy treatment [VAN VLIET et al., 2009]. Antibiotics and also chemotherapeutic intervention are discussed to perturb the indigenous biota to allow consistent enteric infection by a variety of pathogens including *Salmonella enterica*, *Vibrio cholerae*, *Escherichia coli*, *Enterococcus faecalis* as well as *Clostridium difficile*, may resulting in severe gastrointestinal disturbances like *Clostridium difficile*-associated diarrhoea (CDAD) [CROSWELL et al., 2009, SCHALK et al., 2009, VAN VLIET et al., 2009]. *Clostridium difficile* and the emergence in patients receiving chemotherapy will be discussed later.

4.3 BACTERIAL COMMUNITIES

The distal human intestine represents an anaerobic bioreactor programmed with an enormous population of bacteria, dominated by relatively few divisions that are highly diverse at the strain/subspecies level [BACKHED et al., 2005]. Members of nine bacterial phyla were found to inhabit the human gastrointestinal tract of which *Firmicutes*, *Bacteroidetes* and *Actinobacteria* are dominant. Only a limited number of individuals have yet been subjected to the analysis of intestinal microbial diversity and due to individual differences, it is still difficult to define a 'normal and healthy' composition of the microbiota [RAJILIC-STOJANOVIC et al., 2007]. However, diversity is generally thought to be desirable for ecosystem stability and resilience [BACKHED et al., 2005].

The following part will focus on the *Bacteroides*, bifidobacteria and *Clostridium* cluster IV and XIVa group as well as on *Clostridium difficile*, which are subject of this work.

4.3.1 *Bacteroides*

The *bacteroides* subgroup is one of the major populations within the gut community and is characterized as obligate anaerobic, gram-negative and non-sporulating [OHKUMA et al., 2002]. These bacteria have significant effects on human health, most notably in carbohydrate fermentation and catabolism of polysaccharides. Some strains are important opportunistic pathogens, such as *B. fragilis* [LI et al., 2009]. *Bacteroides* species have been reported to show high variations between individuals, although *Bacteroides thetaiotaomicron* is found in all humans [ZWIELEHNER et al., 2009], is very successful in digesting otherwise indigestible dietary polysaccharides [BACKHED et al., 2005] and some members of this group are fibrolytic and produce butyrate [LISZT et al., 2009]. The impact of butyrate on intestinal health will be described in later. Additionally, the *bacteroides* group is discussed to be part of the 'ageing microbiota' [MARIAT et al., 2009] and seems to be related with obesity, showing that the microbiota of obese mice and humans include fewer *bacteroidetes* [DIBAISE et al., 2008].

4.3.2 Bifidobacteria

Bifidobacteria are Gram positive bacteria belonging to the class *Actinobacteria*. Among the commensal bacteria that are resident in the mammalian GIT, Bifidobacteria represent one of the most numerous "probiotic" groups and are predicted to constitute less than 10%, depending on age and diet [TURRONI et al., 2008]. Stimulation of these bacteria has previously been described after prebiotic intervention with inulin and fructo-oligosaccharides [KOLIDA et al., 2007]. Furthermore, they have been shown to be involved in the prevention of atopic disease [OUWEHAND, 2007], obesity and insulin resistance via enhanced barrier function of the gut epithelium [CANI et al., 2007].

4.3.3 *Clostridium* cluster IV

The predominant intestinal *clostridia* belong mainly to cluster XIVa (*Clostridium coccoides* – *Eubacterium rectale* group) and IV (*Clostridium leptum* group).

Some members of these populations are saccharolytic and/or proteolytic species and are able to metabolize a wide variety of substrates. In the colon they are important in the fermentation and putrefaction of food-derived substances, resulting in various metabolites such as fatty acids and gases [MAUKONEN et al., 2006]. Therefore, members of *Clostridium* cluster IV are known to be involved in beneficial functions, including nutrient absorption and epithelial cell maturation and maintenance [WOODMANSEY, 2007].

4.3.4 *Clostridium* cluster XIVa

As mentioned above, *Clostridium* cluster XIVa is one of two abundantly represented clusters of *Firmicutes* in the human colon [MAHOWALD et al., 2009]. The *Clostridium* cluster XIVa group contains many butyrate-producing strains, including *Roseburia* and relatives that can degrade starch and inulin. Furthermore, important non-butyrate producing members of group XIVa are *Ruminococcus torques* and *R. gnavus*, which are among the primary mucin-degrading organisms [DETHLEFSEN et al., 2006]. Other functionally significant attributes that have been identified for clostridial cluster XIVa species include acetogenesis, utilization of aromatic compounds from the diet and metabolism of linoleic acid but the phylogenetic distribution of these attributes is not yet fully established [FLINT et al., 2007].

4.3.5 *Clostridium difficile*

Clostridium difficile is a Gram-positive, spore forming anaerobic bacterium that can reside asymptotically within the intestinal tract of humans [LAWLEY et al., 2009]. The use of broad-spectrum antibiotics disrupt the ecosystem of the normal colonic biota, and may predispose to dense colonization with *C. difficile*, and lead to *C. difficile* associated diseases (CDAD). Patients on antibiotics, hospitalized patients, those with malignancies, immunosuppressed patients and the elderly are all prone to develop CDAD [BALAMURUGAN et al., 2008]. This bacterium is thought to be the causative agent in up to 20% of antibiotic associated disease (AAD) patients. Most of the cases of AAD are thought to be

due to a disturbance of the intestinal microbiota by antibiotics, which are associated with loss of colonization resistance, changes in carbohydrate digestion and production of short-chain fatty acids, altered metabolism of bile acids and changes in both the mucosal and systemic immune system [KONING et al., 2008]. *Clostridium difficile* seems to be a severe complication of aggressive chemotherapy too [SCHALK et al., 2009]. In this context the incidence of *Clostridium difficile* was analyzed in patients receiving chemotherapy and will be subsequently explained in greater detail.

4.4 MICROBIOTA CHANGES WITH AGE

The intestinal microbiota is relatively stable throughout adult life and has been extensively studied; recent studies indicate that modifications and structural changes occur from infants to elderly individuals [MARIAT et al., 2009].

Diet is an important determinant of bowel function and age-related changes in the amounts and types of food consumed caused by a decline in taste and smell, masticatory dysfunction and swallowing difficulties may be important factors contributing to alterations in the gut ecology in old age [BARTOSCH et al., 2004]. Additionally, immunological changes occur in the body with advancing age. Other age related shifts, such as decreased gastric acid secretion and increased mucosal permeability in the gut, have been linked to increases in circulating antibodies to bacteria in the microbiota in elderly subjects [HOPKINS et al., 2001]. Therefore, the inflammatory status of old age can be nurtured from intestinal environment [GUIGOZ et al., 2008]. All this together enables new bacterial populations to exploit novel ecological niches, thereby changing the composition and activities of the microbiota [HOPKINS et al., 2001].

With ageing, a decline in counts of *Bacteroides* with increased age [WOODMANSEY, 2007] was reported and the species diversity within the genus *Bacteroides* is reportedly reduced in elderly compared with the healthy adults [ZWIELEHNER et al., 2009]. As mentioned above, *Bacteroides* species

are nutritionally versatile and changes within this bacterial community could have considerable consequences for the elderly host. In conjunction with the shifts observed in the genus *Bacteroides*, the decline in beneficial bifidobacteria is one of the most marked changes in the elderly gut [WOODMANSEY, 2007]. The majority of studies in the literature report a decrease of beneficial *Lactobacilli* [ZWIELEHNER et al., 2009] and an increase in *Clostridia* in elderly subjects, particularly following antibiotic therapy [WOODMANSEY, 2007, ZWIELEHNER et al., 2009].

These changes of the aged may favour gastrointestinal infections that are frequent in the elderly, including *Clostridium difficile*-associated diarrhoea, what has become a major problem in the elderly hospitalized and nonhospitalized patient [HEBUTERNE, 2003].

Figure 1 shows an overview of the key changes to intestinal microbiota observed in elderly populations [WOODMANSEY, 2007].

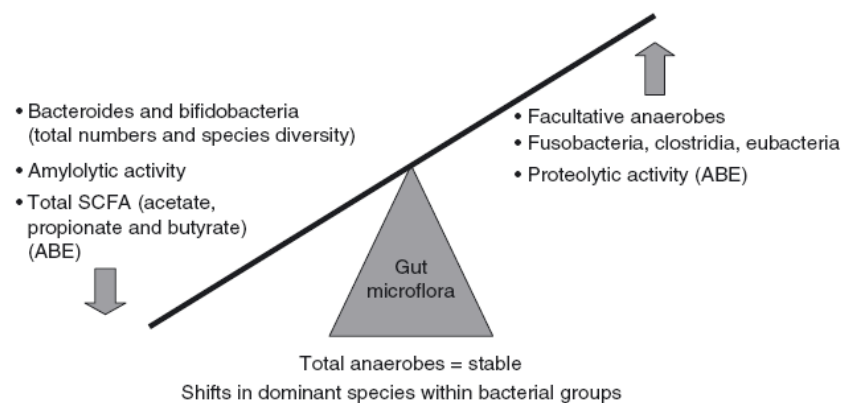


Figure 1 Changes of the GI microbiota in elderly populations
(ABE = antibiotic-treated elderly)

4.5 SHORT CHAIN FATTY ACIDS, BUTYRATE

Short-chain fatty acids (SCFAs), primarily acetate, propionate and butyrate, are organic acids produced within the intestinal lumen by bacterial fermentation or mainly undigested dietary carbohydrates.

An important SCFA produced is butyrate that besides being an energy source for the epithelial cells also influences a wide array of cellular functions affecting colonic health. As such, butyrate may have an anticarcinogenic and anti-inflammatory potential and affect the intestinal barrier and play a role in satiety and oxidative stress [HAMER et al., 2008, HAMER et al., 2009]. Frequently described are the effect on gene expression because of the inhibition of histone deacetylase and suppression of NF- κ B activation [HAMER et al., 2008]. Cell culture studies have indicated that the presence of butyrate at physiological concentrations enhances the growth of normal enterocytes and inhibits that of malignant ones [MCORIST et al., 2008]. In contrast, a few animal and in vitro studies demonstrate negative effects at higher butyrate concentrations on permeability and visceral sensitivity of the large intestine [HAMER et al., 2008]. Thus, the relative and physiologic production rates of these SCFA provide a potentially important link between diet and colonic health and disease [BARCENILLA et al., 2000].

The human colon harbours butyrate-producing species of several genera such as *Clostridium*, *Eubacterium* and *Fusobacterium* [ZWIELEHNER et al., 2009]. Barcenilla et al. (2000) showed that 80% of butyrate-producing isolates of human faecal origin belonged to the clostridial phylogenetic cluster XIVa [MAUKONEN et al., 2006].

A screen of 38 butyrate-producing bacteria from the human colon revealed that the Butyryl-CoA CoA-transferase pathway is the predominant route of butyrate formation in bacteria adapted to live within gut ecosystems, as they use the Butyryl-CoA CoA-transferase gene for the last step of butyrate formation

[LOUIS et al., 2007]. Therefore, abundance of this gene was analyzed by Hippe *et al.* in faecal samples of elderly, young omnivores and vegetarians.

In order to link this section with the 'ageing microbiota' and the 'microbiota in times of immunosuppression (chemotherapy and antibiotics)', studies of bacterial fermentation products in their faeces have shown a decrease in SCFA production in elderly people, associated with a decrease in the number of *Bacteroides* and bifidobacteria. A recent study reveals a close relationship between the decrease in butyrate-producing bacteria and antibiotic-associated diarrhoea, suggesting that a decline of SCFAs may also play a role [ROY et al., 2006].

4.6 MICROBIOTA IN IMMUNODEFICIENCY, E. G.: CHEMOTHERAPY, ANTIMICROBIAL THERAPY

Changes of the microbiota are also observed in pathological conditions such as infections, antibiotic therapy, anti-acid therapy and immune suppression [SCHIFFRIN et al., 2002].

Infections and inflammatory complications remain among the most-encountered serious complications of chemotherapy treatment among patients with cancer, accounting for morbidity and mortality, despite the use of prophylactic antibiotics [VAN VLIET et al., 2009]. Chemotherapy damages the rapidly generated mucosal cells of the gastrointestinal tract, causing diarrhoea or constipation and creating a source for bacterial invasion [GIBSON et al., 2006, NYHLEN et al., 2002]. For this reason 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 100% of patients receiving high dose chemotherapy and stem cell or bone marrow transplantation exhibit abdominal pain, ulceration, bloating, vomiting and diarrhoea typically associated with mucositis, especially large intestine mucositis [STRINGER et al., 2007, STRINGER et al., 2009]. In addition, chemotherapy is reported to destroy the brush border enzymes, which are

responsible for the digestion of both carbohydrates and proteins, possibly affecting the intestinal butyrate production. Bacterial overgrowth has also been reported to be a significant cause of clinical diarrhoea; hence, the damage to crypts leads to the risk of opportunistic infections [GIBSON et al., 2006].

Accompanying antibiotic treatment carries both benefit and risk [CROSWELL et al., 2009]. Antibiotic therapy may influence the human intestinal microbiota, resulting in induction of antimicrobial resistance, overgrowth of pathogens like *Clostridium difficile* and reduction in colonization resistance. Several antineoplastic drugs are known to have an antimicrobial effect [NYHLEN et al., 2002].

Previous studies have investigated the effect of chemotherapeutics and antibiotics on intestinal microbiota, but still little is known about the direct effects of cytostatic drugs on the intestinal microbiota of humans [NYHLEN et al., 2002]. For this reason, we analyzed changes of predominant bacteria in human faeces as a response to chemotherapeutic and antibiotic intervention.

4.7 THE MICROBIOTA AS THERAPEUTIC TARGET

Recent increased interest in an influence of intestinal microbiota on human health and disease resulted in attempts of improving optimally its composition by using probiotics, prebiotics and combinations of both, synbiotics [TLASKALOVA-HOGENOVA et al., 2004].

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit to the host” [NEISH, 2009]. In addition to bacterial strains *Lactobacillus* and *Bifidobacter*, also some non-pathogenic strains of *E. coli* proved suitable for this purpose [TLASKALOVA-HOGENOVA et al., 2004]. Clinical evidence indicates that probiotics are effective in the treatment or prevention of acute viral gastroenteritis, postantibiotic-associated diarrhea, certain pediatric allergic disorders, necrotizing enterocolitis and inflammatory bowel disease (IBD) [NEISH, 2009].

“A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one of a limited number of bacteria in the colon, and thus improves host health” [MACFARLANE *et al.*, 2006]. These complex carbohydrates (inulin, oligofructose) can increase concentrations of SCFAs, including butyrate and indirectly modify immune functions [NEISH, 2009].

Finally, although it might be challenging to select save probiotics that would improve the ecological health of the microbiota, it might be more immediately practical to provide supplementary bacteria to restore a deficiency following treatment with antibiotics and maybe chemotherapy too. This would prevent the expansion of opportunistic pathogens and stimulate cytoprotective responses [NEISH, 2009].

5 MATERIAL AND METHODS

This chapter will focus on study design, materials and methods used for the work “Changes in human faecal microbiota due to chemotherapy analyzed by TaqMan-PCR and PCR-DGGE fingerprinting” by Lassi *et al.*. The same molecular techniques were used in the works of Zwieler *et al.* and Hippe *et al.*, excluding the HPLC-RI method and the use of degenerate primer sets. For detailed information, these methods are described in the attached manuscript by Hippe *et al.*.

5.1 MICROBIOTA DURING CHEMOTHERAPY AND ANTIBIOTIC TREATMENT

Figure 2 shows a schematic overview of the study design and the used methods for assessing the bacterial composition of patients receiving chemotherapy. Afterwards every single step will be described in more detail.

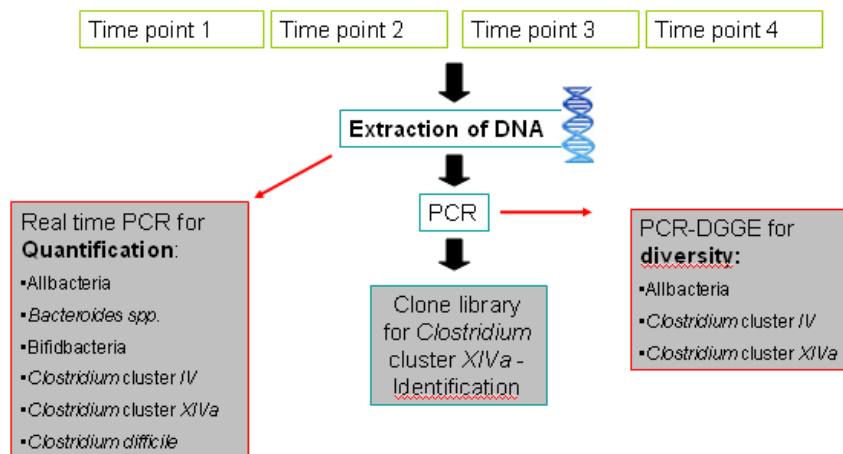


Figure 2 Overview of study design and used methods for this thesis

5.1.1 Study participants and study design

Eleven subjects receiving chemotherapy $\pm$ 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 before or after the onset of treatment within two weeks. The term “out of chemotherapy” is used in the following sections for samples that are currently not under chemotherapeutical intervention. Two out of nine patients had never received any chemotherapy before, while the others had a longer history of chemotherapy. Stool samples of healthy individuals were collected once.

All study participants were interviewed 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 for at least three months period before the collection of faecal samples. One subject receiving chemotherapy additionally suffered from rheumatism while another subject suffered from diabetes mellitus type II, hypertension and obesity. Study populations were gender balanced, with 55% females in both, oncology patients and healthy controls.

Furthermore, patients' medical records reported chemotherapeutic and antimicrobial treatment. All subjects gave written informed consent. The study was approved by the ethics committee of Vienna.

5.1.2 Extraction of bacterial DNA

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 (QIA-GEN) following the manufacturer's protocol. Finally we stored the DNA at -20°C until the analysis was conducted.

5.1.3 Type strains

Type strains, knowing to be a part of the human gastrointestinal microbiota and cloned sequences were used to design a DGGE standard lane marker. Type strains *Bacteroides thetaiotaomicron* DSM 2079T, *Enterococcus faecium* DSM 20477 T, *Lactobacillus reuteri* ATCC 55730 T, *Bifidobacterium longum* ssp. *longum* DSM 20097 T, *Escherichia coli* IMBH 252/07 and clones CL16 and CC34 were used creating a comparable standard lane marker for DGGE gels analyzing bacteria.

5.1.4 Cloning

To create a standard lane marker for DGGE analysis and identifying dominant members of the *Clostridium* cluster XIVa we constructed a clone library from faecal samples. Therefore 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. We picked forty clones, amplified with primer pair T7/Sp6 (Promega) and checked fragment length on 2% agarose gel before clone inserts were sequenced by 'DNA confidence' (Vienna). Finally, nucleotide sequences were corrected for primer and vector sequences in CodonCodeAligner (www.codoncode.com) and

taxonomically identified using 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].

5.1.5 Polymerase chain reaction (PCR)

PCR was carried out amplifying 16S rRNA gene sequences from bacteria in faecal samples, type strains and 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 MgCl₂, 500 nmol/L of both primers and 2 µl of template DNA. When amplifying faecal samples, bovine serum albumin (Fermentas) was added to a final concentration of 400 µg/ml. We used a Robocycler (Stratagene) for all amplifications.

5.1.6 Diversity measurement – PCR-DGGE

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 polyacrylamide gels with a denaturing gradient of 30-60% for predominant bacteria, 30-50% for *Clostridium* cluster IV and 35-50% for *Clostridium* cluster XIVa using a gradient mixer (Hoefer SG 30) and a peristaltic pump. Electrophoresis was performed for 9 h at 130 V at 60°C (predominant bacteria), 5 h at 200 V 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 gradient-variations within one gel.

Target organism	Primer pairs	Sequence (5' - 3')	Ann. temp (°C)	Reference
Bacteria	341F-GC	CCT ACG GGA GGC AGC AG	55	[MUYZER et al., 1993]
	518-R	ATT ACC GCG GCT GCT GG	55	[NEEFS et al., 1991]
<i>Clostridium</i> cluster IV	sg-Clept-F-GC	GCA CAA GCA GTG GAG T	55	[MATSUKI et al., 2004]
	sg-Clept-R	CTT CCT CCG TTT TGT CAA	55	
<i>Clostridium</i> cluster XIVa	Ccocc-F-GC	AAA TGA CGG TAC CTG ACT AA	50	[MATSUKI et al., 2004]
	Ccocc-R	CTT TGA GTT TCA TTC TTG CGA A	50	

Table 1 Primers for PCR-DGGE fingerprinting of 16S rRNA coding regions

5.1.7 Quantitative measurement – TaqMan-qPCR

The abundance of bacteria and bacterial subgroups was measured by 16S rRNA gene-targeting with TaqMan detection. 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. Briefly, 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 in a final volume of 10µl see table 2) and 2µl of bacterial DNA. Amplification programs included an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation step at 95 °C for 30 s, primer annealing at 55 °C (bacteria, *Clostridium* cluster IV), 56 °C (*Clostridium* cluster XIVa), 58 °C (*Clostridium 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 *Clostridium 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 *Clostridium difficile*, using the nanodrop method and calculated DNA copies/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].

Sensitivity of PCR reactions was determined with stepwise dilutions of standard curve DNA. The specificity was confirmed using non-target DNA. All samples were measured in double approach and the average was used for calculation.

Target organism	Primer and probe	Sequence (5' - 3')	Size (bp)	Conc. (nM)	Reference
Bacteria	BAC-338-F	ACT CCT ACG GGA GGC AG	468	1000	[YU et al., 2005]
	BAC-805-R	GAC TAC CAG GGT ATC TAA TCC		1000	
	BAC-516-P	(6-FAM)-TGC CAG CAG CCG CGG TAA TAC-(BHQ-1)		200	
<i>Bacteroides</i>	AlIBac296f	GAG AGG AAG GTC CCC CAC	106	300	[LAYTON et al., 2006]
	AlIBac412r	CGC TAC TTG GCT GGT TCA G		300	
	AlIBac375Bhqr	(6-FAM)-CCA TTG ACC AAT ATT CCT CAC TGC TGC CT-(BHQ-1)		100	
Bifidobacteria	Fwd primer	GCG TGC TTA ACA CAT GCA AGT C	125	300	[PENDERS et al., 2005]
	Rev primer	CAC CCG TTT CCA GGA GCT ATT		300	
	Probe	(6-FAM)-TCA CGC ATT ACT CAC CCG TTC GCC-(BHQ-1)		150	
<i>Clostridium</i> cluster IV	sg-Clept-F	GCA CAA GCA GTG GAG T	239	400	[MATSUKI et al., 2004]
	sg-Clept-R	CTT CCT CCG TTT TGT CAA		400	
	Clept-P ^a	(FAM)-AGG GTT GCG CTC GTT-(BHQ-1)	538	200	
<i>Clostridium</i> cluster XIVa	195-F	GCA GTG GGG AAT ATT GCA		500	[MEIER et al., 1999]
	Ccocc-R	CTT TGA GTT TCA TTC TTG CGA A		500	
	Ccocc-P	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)		150	
<i>Clostridium difficile</i>	Cdiff-F	TTG AGC GAT TTA CTT CGG TAA AGA	151	1000	[PENDERS et al., 2005]
	Cdiff-R	TGT ACT GGC TCA CCT TTG ATA TTC A		1000	
	Cdiff-P	(6-FAM)-CCA CGC GTT ACT CAC CCG TCC G-(BHQ-1)		200	

Table 2 Primers and TaqMan-probes targeting 16S rRNA coding regions of faecal bacteria for absolute and relative quantification used in this study

5.1.8 Statistical analysis

Statistical evaluation of differences between groups (chemotherapy and control) and changes within the chemotherapy group (all time points during chemotherapy, time points after chemotherapy or out of chemotherapy) was carried out using the OriginPro version 8 (OriginLab, Northampton, MA). For

two group comparisons of independent ordinal and interval values the two-sample-T-test and the non-parametric Mann-Whitney-U-test were used. For the analysis of related data the paired-sample-T-test or the non parametric Wilcoxon-signed-ranked-test were used. P values below 0.05 were considered statistically significant.

PCR-DGGE bands were analyzed in GelComparII (www.applied-maths.com) and compared 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. Clustering was performed applying Dice coefficient and UPMGA dendrograms. 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.

5.2 AGEING RELATED SHIFTS OF THE MICROBIOTA

The aim of the study by Zwiehler et al. was to investigate ageing-related shifts in diversity and composition of bacteria, *Bacteroides*, bifidobacteria and *Clostridium* cluster IV by comparing industrialized elderly (n=17, 78-94 years) and young individuals (n=17, 18-31 years). Therefore, qPCR with TaqMan-detection and PCR-DGGE fingerprinting methods were used.

5.3 BUTYRATE

The work by Hippe et al. aimed to detect differences in butyrate production by Buryryl-CoA CoA-transferase gene analysis in faecal samples and measuring butyrate concentration via HPLC-RI. The study population consisted of industrialized elderly (n=17, age=86±8), young omnivores (n=17, age=24±2.5) and young vegetarians (n=16, age=26±5).

6 RESULTS

Results and data of all three described parts of this thesis will be presented in the following chapter. Whereas the aging- and butyrate-results are presented in short, this chapter is principally concerned with the chemotherapy-results, as my research focused on this issue.

6.1 MICROBIOTA DURING CHEMOTHERAPY AND ANTIBIOTIC TREATMENT

6.1.1 Dietary aspects

The participants' dietary habits were assessed 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 (not significant).

6.1.2 qPCR quantification and statistical interpretation

Table 3 shows result from the real time PCR quantification in absolute numbers of bacteria and bacterial subgroups, including mean values and standard deviations for oncology patients and healthy controls.

ON's	Allbac.absolute	Bac.absolute	Bif.absolute	Clept.absolute	Ccocc.absolute	Cdiff.absolute	Unidentified.absolute
ON001/1	1.798.805,94	686.436,18	22.965,45	431.283,01	179.653,46		478.467,84
ON001/2	372.479,33	214.081,92	4.558,69	66.859,19	17.518,96		69.460,57
ON001/3	1.183.463,77	453.079,56	30.777,49	349.670,05	81.729,38		268.207,30
ON001/4	4.900.917,36	1.757.221,14	69.416,26	894.221,86	492.005,88		1.688.052,22
ON002/1	20.212.193,78	5.354.904,16	25,84	1.727.067,70	8.456.716,97		4.673.479,10
ON002/2	23.903.696,36	4.005.111,16	366,48	1.926.246,84	6.273.075,75		11.698.896,13
ON002/3	3.129.907,77	1.537.813,68	65,15	899.451,34	202.334,40		490.243,20
ON002/4	42.134.076,47	8.601.362,67	266,24	6.391.142,51	11.186.629,09		15.954.675,96
ON003/1	5.252.430,48	330.458,04	303.812,95	1.584.639,87	703.331,31		2.330.188,31
ON003/2	32.539.656,72	7.049.121,86	1.322.331,64	11.554.656,05	10.912.078,21		1.701.468,96
ON003/3	8.776.038,82	1.914.216,02	68.520,97	1.613.986,64	3.585.565,52		1.593.749,67
ON003/4	34.133.421,73	5.633.416,81	368.271,38	6.935.425,53	17.973.732,55		3.222.575,46
ON004/1	10.176.085,75	3.675.199,65	123.237,25	2.790.373,95	1.828.490,92		1.758.783,98
ON004/2	2.041.417,98	124.427,95	6.748,31	1.044.114,49	456.546,61		409.580,63
ON004/3	9.397.403,84	2.436.596,74	150.717,25	2.484.676,03	1.113.862,60		3.211.551,22
ON004/4	17.611.326,10	3.143.896,40	388.478,41	5.200.829,47	1.762.498,85		7.115.622,98
ON005/1	58.606.024,90	7.574.676,76	1.485.218,67	8.089.718,24	10.498.104,44		30.958.306,80
ON005/2	77.914.671,15	9.930.958,08	4.295.028,01	24.053.291,85	25.121.947,29		14.513.445,92
ON005/3	47.867.038,82	10.669.686,99	2.232.338,50	8.288.562,23	21.327.802,26		5.348.813,62
ON005/4	45.546.066,19	8.990.544,45	1.150.309,59	11.197.726,83	23.100.668,54		1.106.816,77
ON006/1	4.254.777,75	1.117.961,90	150.748,62	648.065,08	1.921.015,97		416.986,18
ON006/2	19.663.552,55	4.064.824,71	143.528,56	1.769.516,54	4.984.825,07		8.700.857,66
ON006/3	5.817.626,36	1.692.997,14	126.902,02	1.911.000,59	1.832.499,43		254.227,18
ON006/4	11.491.330,22	2.619.686,08	354.166,89	1.450.849,53	3.690.397,55		3.376.230,17
ON007/1	32.882.071,40	13.715.080,97	897,46	3.410.583,24	7.731.920,78		8.023.588,95
ON007/2	14.010.666,45	2.027.428,84	1.032,00	2.671.080,63	1.991.983,19		7.319.141,79
ON007/3	31.475.072,44	16.519.292,63	823,50	1.586.561,05	13.562,81		13.354.832,44
ON007/4	210.972,37	82.379,99	34,45	55.314,07	9.572,75		63.671,12
ON008/1	20.526.835,88	5.611.144,84	718,75	1.247.117,30	7.680.688,02		5.987.166,97
ON008/2	44.425.634,50	16.318.239,73	895,78	3.476.321,68	15.493.685,21		9.136.492,11
ON008/3	25.709.141,44	11.964.895,51	718,60	2.268.109,88	5.119.868,83		6.355.548,62
ON008/4	4.789.406,38	1.833.583,83	50,22	255.512,39	404.272,01		2.295.987,93
ON009/1	60.928.403,72	19.783.140,36	349.975,84	6.483.641,99	5.835.644,77	6.882,51	28.469.118,25
ON009/2	79.557.247,54	24.331.906,09	426.303,13	7.125.594,64	6.230.655,43	3.410,13	41.439.378,12
ON009/3	18.692.507,80	7.732.250,88	2.051,63	1.534.255,90	6.614.636,98	228.486,37	2.580.486,05
ON009/4	22.422.312,29	7.987.608,92	13.249,55	1.508.579,53	2.774.425,14	90.328,31	10.048.120,85
ON010/1	13.477.917,70	2.837.015,05	73,34	1.122.511,68	1.498.517,07		8.019.800,57
ON010/2	18.520.677,78	2.213.914,71	15,13	830.143,08	993.439,98		14.483.164,88
ON010/3	52.591.368,06	5.899.979,48	3.869,15	2.670.649,71	4.623.410,70		39.393.459,02
ON010/4	21.962.667,14	5.147.834,94	1.465,47	1.149.697,73	2.797.644,59		12.866.024,42
ON011/1	44.187.524,40	6.037.882,96	441.875,24	2.509.998,68	2.374.357,37		32.823.410,14
ON011/2	9.505.774,72	865.600,46	95.057,75	1.175.348,89	2.030.535,80		5.339.231,83
ON011/3	4.652.268,06	1.640.708,80	0,05	180.029,67	397.823,15	181.438,45	2.252.267,92
ON011/4	30.489.412,48	5.277.219,74	0,29	32.637,34	292.051,98	243.915,30	24.643.587,83
mean value	23.630.510,40	5.713.767,93	321.316,09	3.286.296,92	5.286.630,17	125.743,51	9.005.352,45
standard deviation	20.746.842,40	5.581.373,91	766.855,30	4.329.980,87	6.472.251,56	107.675,65	10.688.197,15
C's	Allbac.absolute	Bac.absolute	Bif.absolute	Clept.absolute	Ccocc.absolute	Cdiff.absolute	Unidentified.absolute
C001	67.127.580,45	21.630.083,97	1.104.596,65	3.016.391,93	22.372.916,47		19.003.591,43
C002	126.948.085,08	19.212.984,02	2.462.247,54	9.444.500,94	68.718.443,26		27.109.909,32
C003	65.278.056,00	25.196.457,64	472.238,95	1.610.808,79	14.988.708,20		23.009.842,41
C004	22.910.238,96	3.611.549,33	121.147,51	750.523,91	6.857.928,70		11.569.087,50
C005	47.074.467,77	18.138.079,80	353.519,38	1.308.964,14	12.538.810,79		14.735.093,66
C006	80.856.946,54	4.609.927,63	105.023,05	12.371.776,73	54.236.684,90		9.533.534,22
C007	58.912.801,05	10.278.138,84	527.253,76	8.869.928,30	37.824.150,42		1.413.329,73
C008	137.945.616,44	15.770.084,32	510.575,47	34.278.520,13	5.002.288,46		82.384.148,07
C009	30.738.626,46	10.320.149,60	576.296,72	2.839.348,81	3.367.532,31	1.807,64	13.633.491,38
mean value	70.865.824,08	15.612.467,88	722.274,14	11.778.939,46	40.454.085,96	1.807,64	22.488.003,08
standard deviation	39.383.963,12	6.599.890,79	703.559,31	15.979.257,31	60.636.038,36		23.693.396,92

Table 3 Absolute numbers of bacteria and bacterial subgroups

Figure 3 illustrates the 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), *Clostridium difficile* (Cdiff) and unidentified bacteria analyzed by TaqMan-qPCR

are shown (Figure 3). The microbiota composition of every patient receiving chemotherapy (ON) and control individual (C) is shown in detail. The absolute numbers are related to 2µl of faeces extract (x 1.000.000). The abbreviation 'ChT' stands for Chemotherapy, 'Ab' for Antibiotics, 'F' stands for fever and 'T' for blood stem cell transplantation. The pattern over the bar chart indicates sampling points during chemotherapy (yellow fields), out of chemotherapy, during antibiotic intervention (gray fields), during fever and when blood stem cell transplantation occurred. Subject ON001 received chemotherapy before sampling.

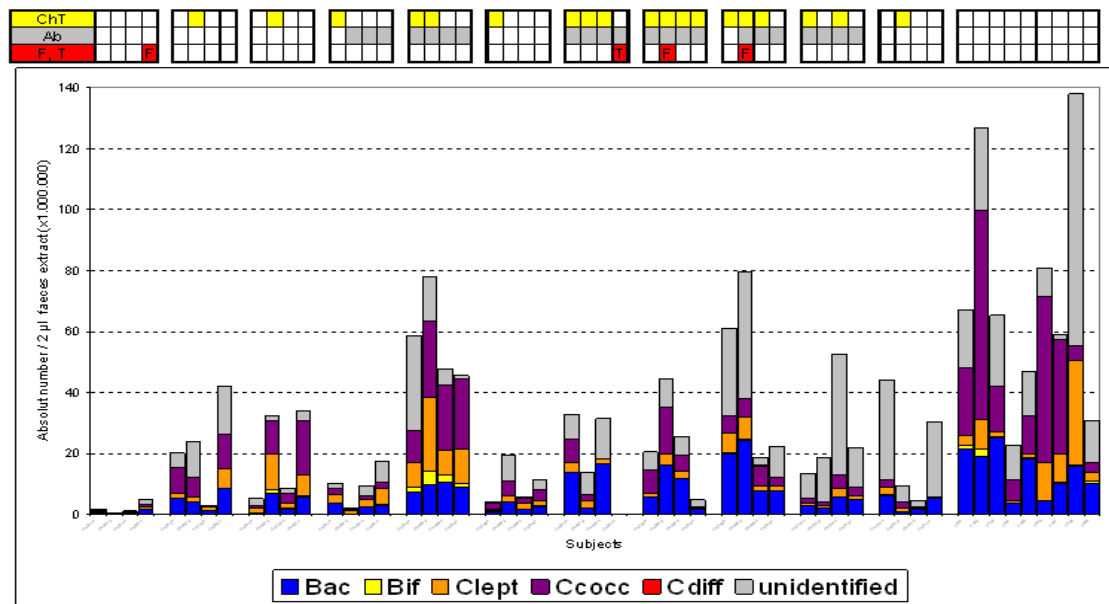


Figure 3 Microbiota composition of the study population in absolute numbers

Patients receiving chemotherapy harboured only $25 \pm 22\%$ of the absolute bacterial load in their faeces compared to healthy individuals. This decrease affected *Bacteroides*, bifidobacteria, *Clostridium* cluster IV and XIVa. Despite high inter- and intraindividual variations, the differences in absolute numbers of bacteria ($p = 0.02$), *Bacteroides* ($p=0.01$), bifidobacteria ($p=0.001$) and *Clostridium* cluster XIVa ($p=0.001$) were statistically significant.

Figure 3 shows that time points after chemotherapeutical intervention often show a decline of all bacterial subgroups, followed by a rebound of bacterial abundance. Subject ON007 shows a sharp decline of bacteria and bacterial

subgroups at time point 4 following medication due to blood stem cell transplantation.

In contrast to absolute numbers, Figure 4 and Table 4 show the quantification as percentage of total bacterial DNA. The mean proportion of *Bacteroides* in stool samples was $27 \pm 12\%$ in chemotherapy patients and $23 \pm 12\%$ in healthy individuals. The mean percentage of bifidobacteria in patients was $1.17 \pm 1\%$ and $1\% \pm 0.7\%$ in controls. Patients harboured $16 \pm 11\%$ of *Clostridium* cluster IV and $20 \pm 14\%$ of *Clostridium* cluster XIVa, while controls harboured $10 \pm 7\%$ and $34 \pm 22\%$ of clostridial clusters IV and XIVa. The red circles in figure 4 highlight the emergence of *Clostridium difficile*.

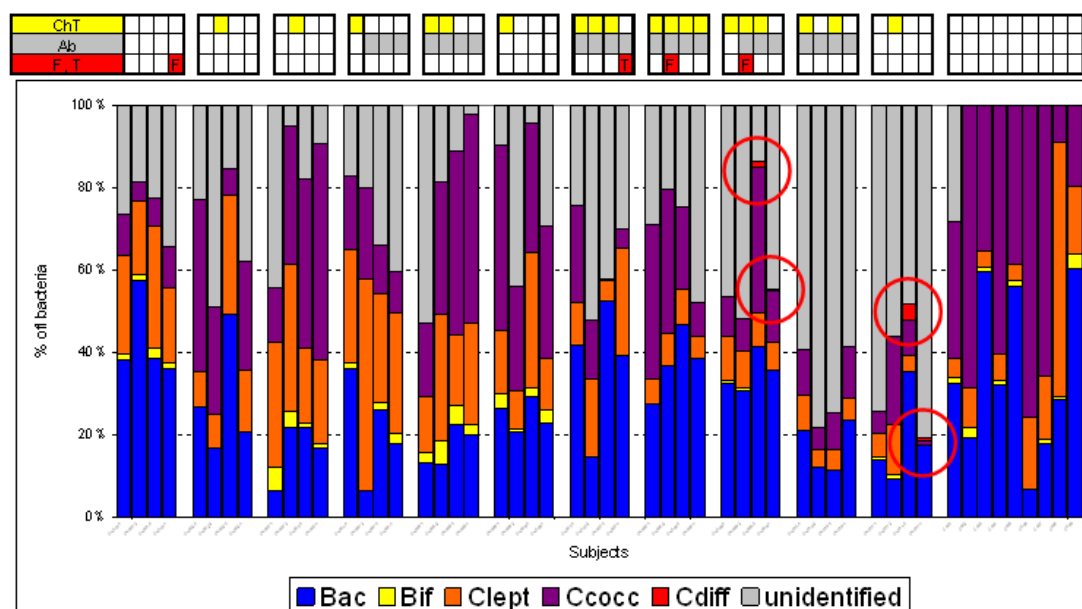


Figure 4 Percentage of bacterial subgroups in relation to analyzed bacteria

18% of oncology patients harboured *Clostridium difficile*. Patient ON009 harboured *C. difficile* at four time points investigated with a mean proportion of $0.4 \pm 0.7\%$, showing the highest level (1.22%) at sampling point 3 (highlighted in Figure 4). *C. difficile* emerged at time point 3 in patient ON011 (3.90% of all analyzed bacteria, highlighted in Figure 4). However, one control individual (C009) harboured *C. difficile* as well.

ON's	Bac%	Bif%	Clept%	Ccocc%	Cdiff%	Unidentified%
ON001/1	38,16	1,28	23,98	9,99		26,60
ON001/2	57,47	1,22	17,95	4,70		18,65
ON001/3	38,28	2,60	29,55	6,91		22,66
ON001/4	35,85	1,42	18,25	10,04		34,44
ON002/1	26,49	0,00	8,54	41,84		23,12
ON002/2	16,76	0,00	8,06	26,24		48,94
ON002/3	49,13	0,00	28,74	6,46		15,66
ON002/4	20,41	0,00	15,17	26,55		37,87
ON003/1	6,29	5,78	30,17	13,39		44,36
ON003/2	21,66	4,06	35,51	33,53		5,23
ON003/3	21,81	0,78	18,39	40,86		18,16
ON003/4	16,50	1,08	20,32	52,66		9,44
ON004/1	36,12	1,21	27,42	17,97		17,28
ON004/2	6,10	0,33	51,15	22,36		20,06
ON004/3	25,93	1,60	26,44	11,85		34,17
ON004/4	17,85	2,21	29,53	10,01		40,40
ON005/1	12,92	2,53	13,80	17,91		52,82
ON005/2	12,75	5,51	30,87	32,24		18,63
ON005/3	22,29	4,66	17,32	44,56		11,17
ON005/4	19,74	2,53	24,59	50,72		2,43
ON006/1	26,28	3,54	15,23	45,15		9,80
ON006/2	20,67	0,73	9,00	25,35		44,25
ON006/3	29,10	2,18	32,85	31,50		4,37
ON006/4	22,80	3,08	12,63	32,11		29,38
ON007/1	41,71	0,00	10,37	23,51		24,40
ON007/2	14,47	0,01	19,06	14,22		52,24
ON007/3	52,48	0,00	5,04	0,04		42,43
ON007/4	39,05	0,02	26,22	4,54		30,18
ON008/1	27,34	0,00	6,08	37,42		29,17
ON008/2	36,73	0,00	7,83	34,88		20,57
ON008/3	46,54	0,00	8,82	19,91		24,72
ON008/4	38,28	0,00	5,33	8,44		47,94
ON009/1	32,47	0,57	10,64	9,58	0,01	46,73
ON009/2	30,58	0,54	8,96	7,83	0,00	52,09
ON009/3	41,37	0,01	8,21	35,39	1,22	13,81
ON009/4	35,62	0,06	6,73	12,37	0,40	44,81
ON010/1	21,05	0,00	8,33	11,12		59,50
ON010/2	11,95	0,00	4,48	5,36		78,20
ON010/3	11,22	0,01	5,08	8,79		74,90
ON010/4	23,44	0,01	5,23	12,74		58,58
ON011/1	13,66	1,00	5,68	5,37		74,28
ON011/2	9,11	1,00	12,36	21,36		56,17
ON011/3	35,27	0,00	3,87	8,55	3,90	48,41
ON011/4	17,31	0,00	0,11	0,96	0,80	80,83
mean value	26,84	1,17	16,22	20,39	1,06	35,22
standard deviation	12,68	1,58	11,01	14,43	1,47	20,72
C's	Bac%	Bif%	Clept%	Ccocc%	Cdiff%	Unidentified%
C001	32,22	1,65	4,49	33,33		28,31
C002	15,13	1,94	7,44	54,13		21,36
C003	38,60	0,72	2,47	22,96		35,25
C004	15,76	0,53	3,28	29,93		50,50
C005	38,53	0,75	2,78	26,64		31,30
C006	5,70	0,13	15,30	67,08		11,79
C007	17,45	0,89	15,06	64,20		2,40
C008	11,43	0,37	24,85	3,63		59,72
C009	33,57	1,87	9,24	10,96	0,01	44,35
mean value	23,16	0,98	9,43	34,76	0,01	31,66
standard deviation	12,55	0,67	7,61	22,51		18,32

Table 4 Relative amount of bacterial subgroups

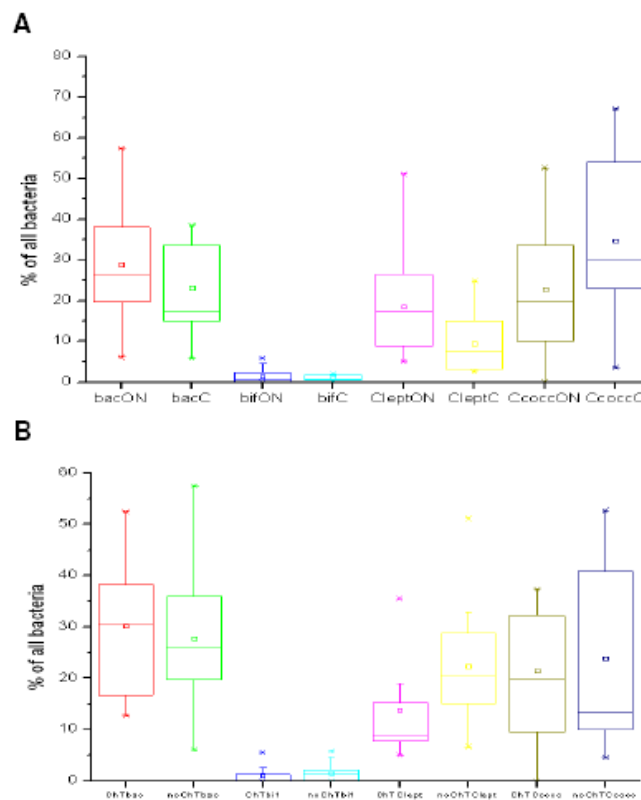


Figure 5 (A) Relative amount of bacteria in oncology patients in comparison to healthy individuals. (B) Comparison of samplings during chemotherapy with samplings out of chemotherapy

Figure 5 A shows differences between groups concerning the subgroups *Clostridium* cluster IV and XIVa. While oncology patients had significantly more ($p = 0.02$) *Clostridium* cluster IV, controls had more *Clostridium* cluster XIVa.

Graph B in figure 4 points out differences in faecal microbiota composition at time points during chemotherapy compared to time points out of chemotherapy. The mean percentage of *Clostridium* cluster IV at time points during chemotherapy was 22 ± 10 %, compared to time points out of chemotherapy 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 $30 \pm 12\%$, $1.2 \pm 1.7\%$ and $22 \pm 11\%$ at time points during chemotherapy and $28 \pm 12\%$, $1.6 \pm 1.5\%$ and $24 \pm 17\%$ at time points out of chemotherapy.

6.1.3 PCR-DGGE diversity assessment and statistical interpretation

The mean number of bacterial bands per patient receiving chemotherapy was 21.8 ± 5 (Figure 6), for *clostridium* cluster IV 9 ± 5 bands (Figure 7) and for *clostridium* cluster XIVa 14.9 ± 7 bands (Figure 8). 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.

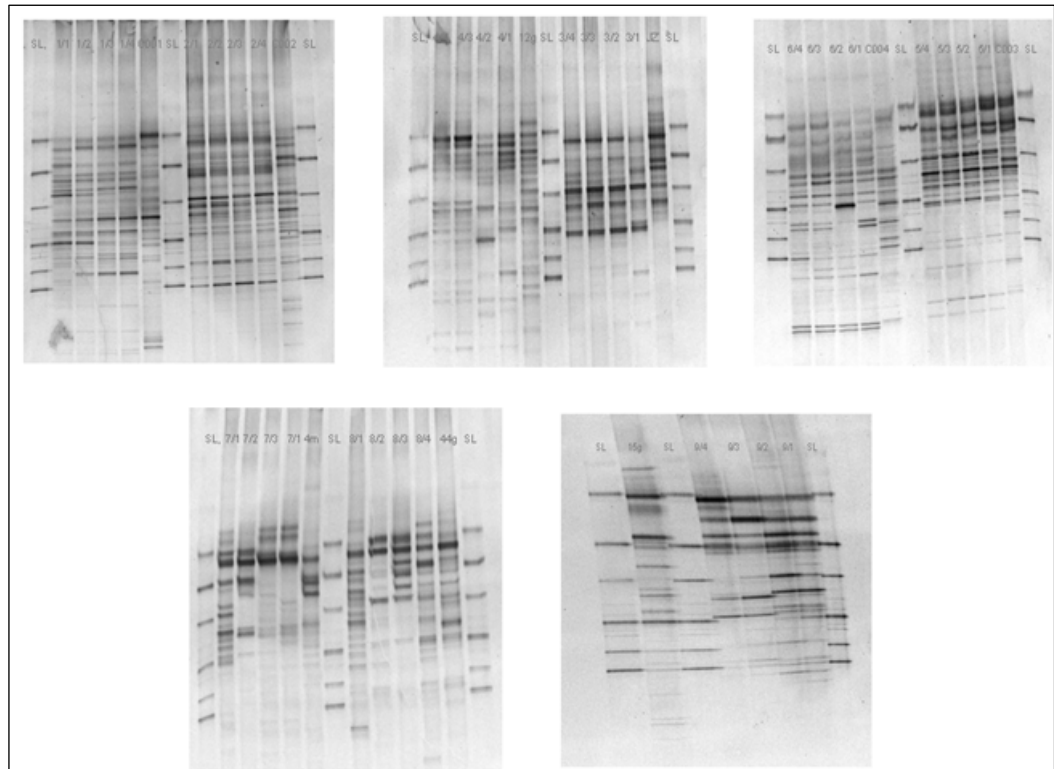


Figure 6 DGGE fingerprinting of bacteria

Figure 7 DGGE fingerprinting of *Clostridium* cluster VI

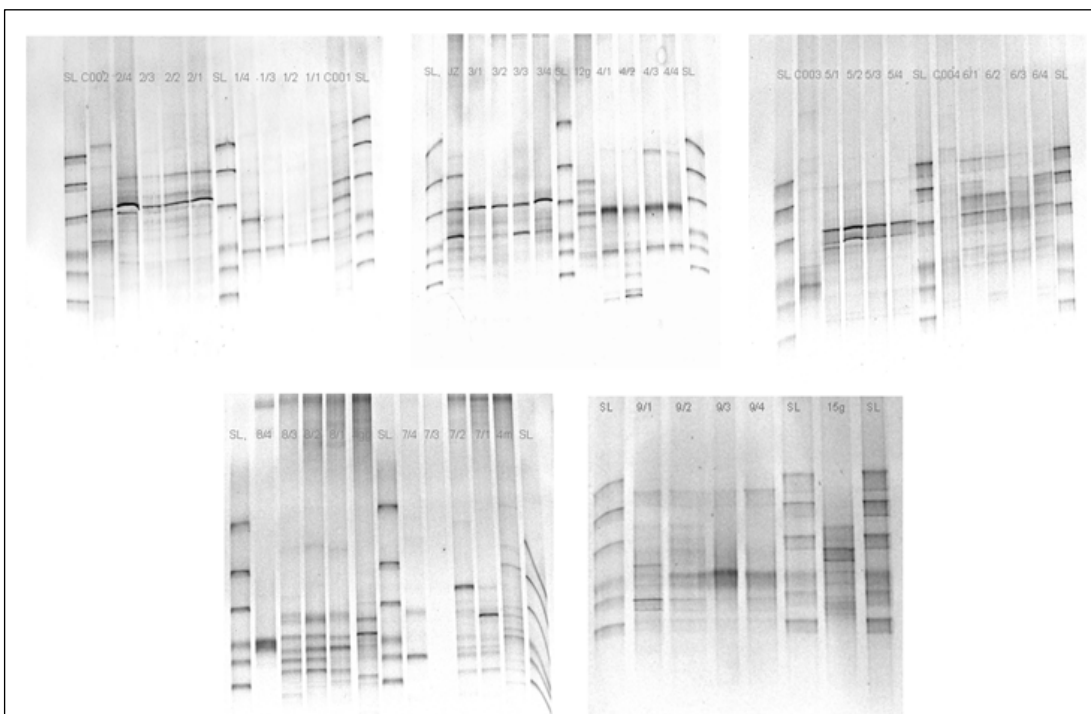


Figure 8 DGGE fingerprinting of *Clostridium* cluster XIVa

The amount of bands as well as Shannon and Simpson diversity indices (Figure 9) show greater diversity of bacteria and *Clostridium* cluster XIVa in controls. In contrast, clostridial cluster IV DGGE fingerprints show greater diversity indices in oncology patients compared to controls. During chemotherapy, a significant ($p = 0.02$ for Simpson index, $p = 0.03$ for Shannon index) decrease in bacterial diversity was seen compared to time points out of chemotherapy (Figure 9).

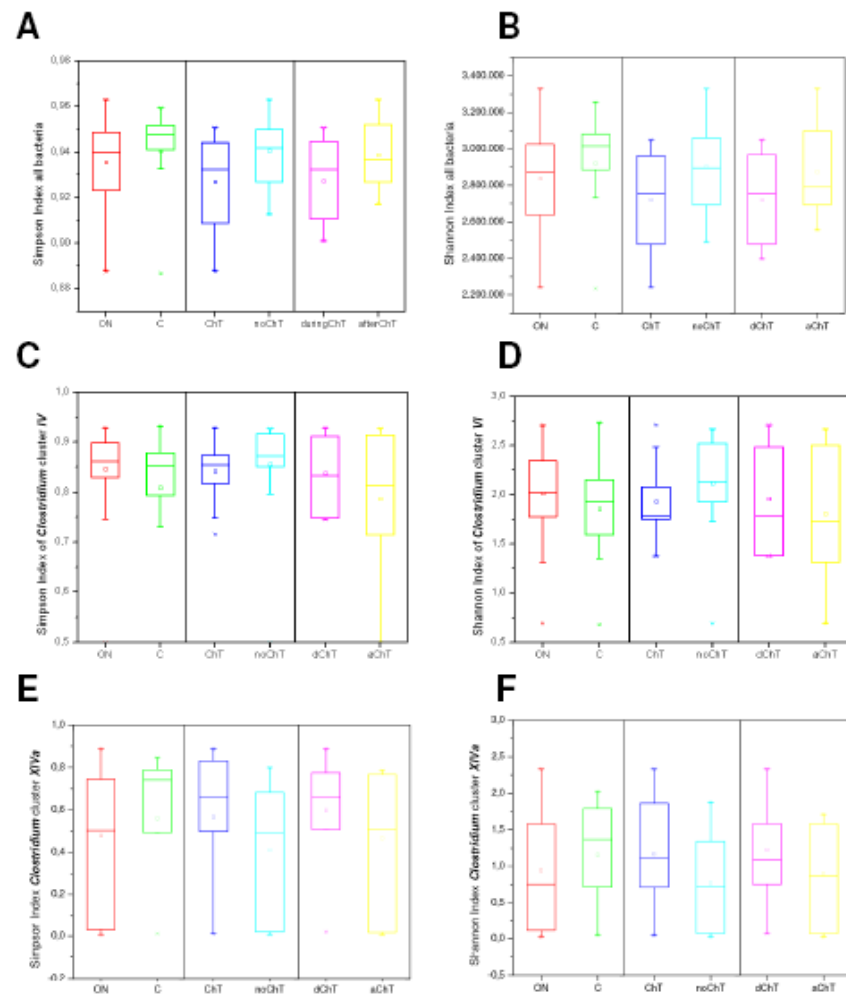


Figure 9 Simpson and Shannon diversity indices

Figure 10 displays PCA of *Clostridium* cluster IV fingerprints. Graph A distinguishes between oncology patients (ON) and healthy control patients (C), graph B indicates separation of time points out of chemotherapy (O), during chemotherapy (T) and control individuals (C) according to their *Clostridium* cluster IV DGGE fingerprints. PCA analysis thus shows distinctive clustering of

O and T samples. The red arrows in the plot showing graph C on the right side tell us that bands 178.16, 169.81 and others are characteristic for O samples. Bands 207.44, 210.53, 208.17, 302.36, 204.66 and 299.79 are characteristic for samples T and responsible for the separation of samples T and O.

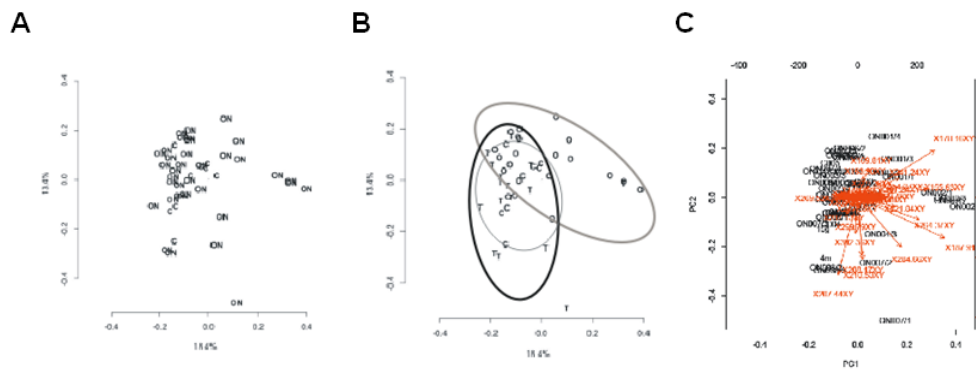


Figure 10 PCA of *Clostridium* cluster IV DGGE fingerprints; Grey circles indicate O, black oval indicates T and grey thin oval indicates control samples

6.2 AGEING RELATED SHIFTS OF THE MICROBIOTA

TaqMan quantification illustrated that elderly harboured only $69\% \pm 21.6\%$ of the total bacterial load compared to controls. Industrialized elderly harboured significantly more *Bacteroides* than young volunteers ($P=0.016$). Despite highly diverse levels of bifidobacteria among samples, the differences between young and elderly study participants were statistically significant ($p=0.026$). Additionally, elderly persons had significantly less members of *Clostridium* cluster IV in their faecal microbiota than young volunteers ($p=0.036$) [ZWIELEHNER et al., 2009]. Figure 11 shows proportions of *Bacteroides*, Bifidobacteria and *Clostridium* cluster IV of the total bacterial load of industrialized elderly and young volunteers. The total amount of bacteria is shown relative to the mean counts for healthy young individuals as previously published by Zwielehner et al..

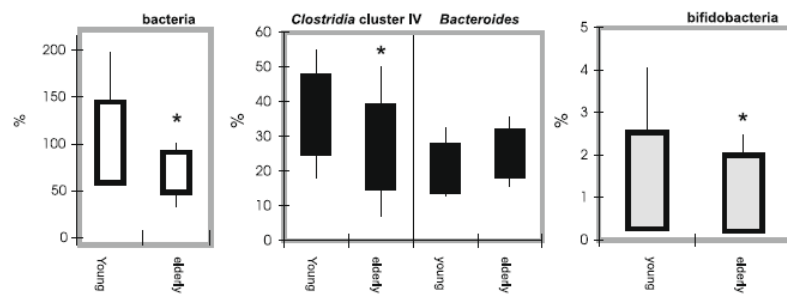


Figure 11 Microbiota composition of industrialized elderly and young individuals [ZWIELEHNER et al., 2009]

DGGE bandpattern analysis of bacteria showed 16.6 ± 3 bands for industrialized elderly and 20 ± 3 bands in controls. An average of 13 bands was observed in individual DGGE bandpatterns of bifidobacteria. Fingerprint analysis of *Bacteroides* indicated 7.6 ± 2.5 bands for elderly, whilst young probands indicated an average of 9.5 ± 3 bands. Diversity indices (Figure 12) showed a tendency to be lower for elderly than for controls. *Clostridium* cluster IV bandpattern analysis yielded an average of 10 ± 3.5 bands for elderly persons and 13 ± 3 bands per young individual. Shannon and Simpson diversity indices (Figure 12) were significantly ($p=0.02$) lower for aged than for the young people [ZWIELEHNER et al., 2009].

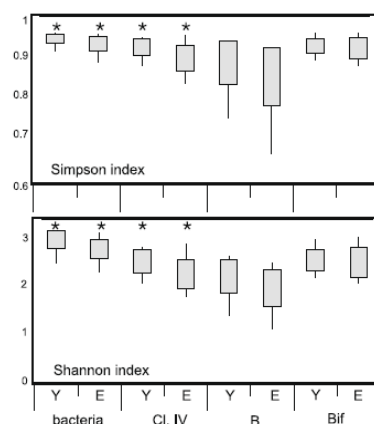


Figure 12 Diversity indices according to Simpson and Shannon of young individuals and elderly persons published by Zwielehner et al.. Y, young; E, elderly; Cl.IV, *Clostridium* cluster IV; B, *Bacteroides*; Bif, bifidobacteria [ZWIELEHNER et al., 2009].

6.3 BUTYRATE

The average butyrate concentration for omnivores measured by HPLC was $0.012136 \pm 27\% \mu\text{mol g}^{-1}$ faeces, for elderly $0.00574 \pm 8.7\% \mu\text{mol g}^{-1}$ faeces and for vegetarians $0.00733 \pm 0.844\% \mu\text{mol g}^{-1}$.

The quantification of Butyryl-CoA CoA-transferase gene indicates significantly higher abundance in vegetarians than in omnivores. Using the CoATD primer set, vegetarians ($7.46 \times 10^8 \pm 2.29 \times 10^8$ copies/ μl) had the highest abundance of the Butyryl-CoA CoA-transferase gene. The abundance was significantly higher compared to omnivores ($5.26 \times 10^8 \pm 2.38 \times 10^8$ copies/ μl ; $p=0.0001$) and elderly ($p=0.01$). The abundance of this functional gene in elderly ($5.26 \times 10^8 \pm 2.38 \times 10^8$ copies/ μl) showed highest variation, being significantly higher than those found in omnivores ($p=0.0007$) but lower than in vegetarians. Quantification with the CTFB primer pair yielded a gene abundance of $4.12 \times 10^8 \pm 1.81 \times 10^8$ copies/ μl for omnivores, $4.6 \times 10^8 \pm 2.51 \times 10^8$ copies/ μl for vegetarians and $3.7 \times 10^8 \pm 1.89 \times 10^8$ copies/ μl for elderly individuals. PCT primers show abundances of $8.61 \times 10^8 \pm 3.12 \times 10^8$ copies/ μl for omnivores, $8.53 \times 10^8 \pm 5.28 \times 10^8$ copies/ μl for vegetarians and $6.55 \times 10^8 \pm 4.73 \times 10^8$ copies/ μl for elderly. This showed a trend for low abundances in elderly and marginally higher abundances in vegetarians, but not significant [HIPPE, 2009].

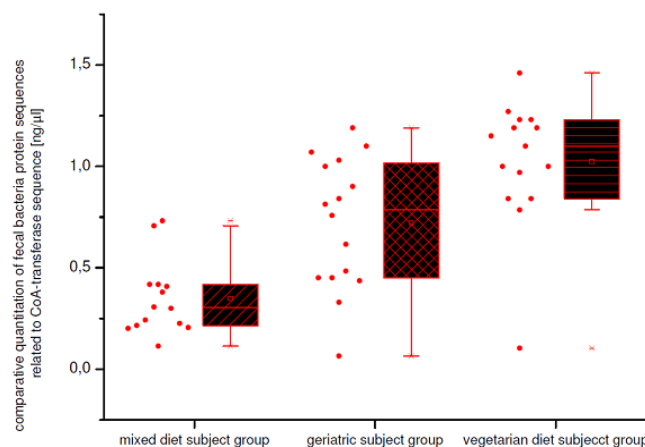


Figure 13 Butyryl-CoA CoA-transferase gene sequences in faeces of elderly, omnivores and vegetarians.

7 DISCUSSION

7.1 METHODS

7.1.1 Study population

Many study participants had a longer history of different chemotherapeutic regimens. Only two individuals had never received any cancer therapy before. Furthermore, the types of cancer and the treatment were variable. Therefore, it can not be hypothesized, which changes occurred at the beginning of treatment cycles. Also patients who did not receive any antibiotics showed similar alterations than those under antimicrobial treatment. In conclusion, it can be hypothesized that chemotherapeutic intervention itself causes severe perturbations of the gastrointestinal microbiota.

7.1.2 Faecal samples

Faecal microbial communities are composed of autochthonous gut members and by transient bacteria; consequently analysis of faecal samples may overestimate the actual composition of the GIT microbiota of humans. Besides faecal samples, also other intestinal samples such as specimens from the human colon, has been determined. Some of these studies suggest that faecal samples do not represent the bacterial community in other parts of the GI tract [ZOETENDAL et al., 2006, ZOETENDAL et al., 2002]. In contrast, a paper by Van der Waaij *et al.* suggested that colonic microbes are not in direct contact with the mucosa and therefore no significant difference was found between colonic biopsies and faeces [VAN DER WAAIJ et al., 2005]. However, 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 do reflect shifts in populations [TURRONI et al., 2008].

7.1.3 Culture- vs. culture-independent methods

In the past, the microbiota of the gut has been monitored by cultivation-based techniques, but limitations of these techniques and the development of more sensitive and accurate molecular detection methods have brought new insights to the field [MATSUKI et al., 2004]. Besides some advantages including the possibility to perform biochemical and physiological investigations, classical techniques are time consuming and laborious. Moreover, the samples to be examined need to be processed immediately while results are greatly affected by media and growth conditions [TURRONI et al., 2008]. For this reason, culture-independent methods on the basis of 16S rRNA genes were used for this study. Currently, PCR with 16SrRNA-based specific primers has been applied to microbiota analyses as the most sensitive and rapid method [MATSUKI et al., 2004].

7.1.4 Real time quantitative PCR (qPCR)

An accurate and successful real time qPCR assay requires great sensitivity, specificity and efficiency. Therefore, sensitivity of all PCR reactions was determined with stepwise dilutions of standard curve DNA and specificity was confirmed using non-target DNA. To ensure a good efficiency, temperature profiles for primer pairs were optimized.

In this study we used type strains *Bacteroides thetaiotaomicron*^T, *Bifidobacterium longum* ssp. *longum*^T and *Clostridium 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 because some species might be preferentially amplified. Therefore, absolute amounts should be considered as semiquantitative.

7.1.5 DGGE

Diversity was assessed using the PCR-DGGE method. Each lane of a PCR-DGGE gel represents a microbial fingerprint of a faecal sample; each band within a lane corresponds to 1 bacterial species, although different species may

sometimes have the same melting behaviour [VAN VLIET et al., 2009]. Several species possess more than one 16S rRNA operon such as *Escherichia coli*^T who forms 4 DGGE bands. The limitations of DGGE in microbial analysis have been previously explored by Muyzer *et al.*. Nevertheless, substantial information about species composition can be obtained from very complex microbial communities by DGGE analysis [MUYZER et al., 1998].

7.2 RESULTS – CHEMOTHERAPY AND ANTIBIOTIC TREATMENT

In this study we investigated how the use of cancer chemotherapy ± antibiotic treatment perturbs the faecal microbial ecosystem over the course of the therapy. The core question was if the microbiota is able to return to its original profile after chemotherapeutic and antibiotic intervention and if *Clostridium difficile* could be detected more often following therapy. Another question was if shifts between bacterial subgroups (*Bacteroides*, bifidobacteria, *Clostridium* cluster IV and XIVa) occurred during the therapy and some bacterial subgroups are more sensitive to chemotherapy than others.

The majority of studies previously done on the effect of chemotherapy on human faecal microbiota used standard microbiological culture techniques [NYHLEN et al., 2002, STRINGER et al., 2007]. Most other studies focused on the colonization with pathogenic bacteria [SCHALK et al., 2009, VAN VLIET et al., 2009] in patients with cancer and chemotherapy-induced diarrhea [ABD EL-ATTI et al., 2009, STRINGER et al., 2007].

The results of the *Bacteroides* analysis are not in agreement with the results of Nyhlén *et al.* who showed increased counts of *Bacteroides* spp. of samples obtained during chemotherapy, but a stable microbiota in most patients. Discrepancies in changes of the *Bacteroides* abundance might be due to differences in detection techniques, as Nyhlén *et al.* used culture-dependent methods. Nyhlén *et al.* also reported significantly increased counts of yeast in patients, making it a focus for further research in immunocompromised patients [NYHLEN et al., 2002].

The results show significant effects of chemotherapy and antibiotic treatment on intestinal microbiota. Despite high individual variations, a significantly lower absolute bacterial load in faeces of patients receiving chemotherapy in comparison to healthy controls is shown. These findings are in line with data from van Vliet *et al.*, who reported 100-fold lower total bacterial numbers during chemotherapy treatment than in healthy controls [VAN VLIET *et al.*, 2009]. 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. Faecal microbiota of patients receiving chemotherapy decreases after cycles of chemotherapy. After the end of chemotherapeutic cycles the magnitude of microbiota recovers within a few days sometimes even showing a “rebound-effect”, supporting the hypothesis that the microbiota is able to recover after chemotherapeutic intervention. 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 but at lower diversity than controls. This suggests that *Clostridium* cluster IV harbours some species that are particularly vulnerable and some that take advantage of disruption by chemotherapeutic intervention.

Furthermore, the incidence of *Clostridium difficile* in subject ON009 and ON011 was accompanied by a decrease of the genera bifidobacteria and *Clostridium* cluster IV. Bifidobacteria and members of *Clostridium* cluster IV might – under undisrupted conditions- hold *Clostridium difficile* at bay, or the higher presence of bifidobacteria enhances the barrier function of the gut epithelium that much that *Clostridium difficile* cannot reproduce. Another possibility is that *Clostridium difficile* overgrows bifidobacteria and members of *Clostridium* cluster IV. These bacterial subgroups (bifidobacteria, *Clostridium* cluster IV and *Clostridium difficile*) might occupy the same niche and Toxin A and B produced by *Clostridium difficile* could suppress bifidobacteria and *Clostridium* cluster IV. But

this phenomenon was only found in two patients. Therefore, more study participants are needed to support this hypothesis.

As mentioned above, some study participants received antibiotics whilst others did not. Also patient who did not receive any antibiotics showed similar alteration than those under antimicrobial therapy. Thus, we conclude that chemotherapeutic intervention itself causes severe perturbations of the gastrointestinal microbiota. Van Vliet *et al.* tested the effect of chemotherapy in vitro and showed a direct bacteriostatic effect of chemotherapeutics on bacterial growth [VAN VLIET *et al.*, 2009].

This study/thesis showed that changes and shifts in human microbiota occur during chemotherapy and that some genera seem to be more affected than others. Further research will have to be done to investigate possible prebiotic interventions for maintaining gut health during chemotherapeutic and/or antibiotic treatment. The emergence of *Clostridium difficile* and the decrease of some genera could be shortened or even avoided.

7.3 RESULTS – AGING MICROBIOTA

To compare patterns in several microbial genera of the gastrointestinal tract between industrialized elderly and young healthy volunteers, a combined molecular approach was used, as previously described in this thesis. PCR-DGGE was used to compare diversity of bacteria, *Clostridium* cluster IV, bifidobacteria and *Bacteroides*. qPCR was used to quantify abundance of these bacterial subgroups [ZWIELEHNER *et al.*, 2009].

Faeces of elderly had less total bacteria and lower bacterial diversity compared to that of the young subjects. Samples of industrialized elderly showed an increase in the relative abundance of *Bacteroides*, although this group displayed less diversity than in young subjects. Significantly higher abundance of bifidobacteria and *Clostridium* cluster IV was shown for young volunteers, also showing a greater diversity [ZWIELEHNER *et al.*, 2009].

Previous studies also reported a reduction of diversity of bifidobacteria associated with ageing [HOPKINS et al., 2002, WOODMANSEY et al., 2004] and hospitalization of the elderly [BARTOSCH et al., 2004]. The elderly study population used for the study by Zwielehner *et al.* was supplemented with soluble fibre, but this prebiotic intervention was apparently not able to antagonize ageing-related shifts in bifidobacteria [ZWIELEHNER et al., 2009].

The elderly study population showed a relative increase in abundance of Bacteroides, whilst others [BARTOSCH et al., 2004, HOPKINS et al., 2002, WOODMANSEY et al., 2004] reported a decrease in the relative abundance of this group. However, all the previous studies reported a decrease of diversity of this group, like the study by Zwielehner *et al.* [ZWIELEHNER et al., 2009].

Reduced numbers of Bacteria in the faeces of elderly may reflect the physiological changes associated with ageing. These include prolonged colonic transit time and reduced dietary energy requirement and food uptake. Furthermore, reductions of important genera like *Clostridium* cluster IV and bifidobacteria might result in decreased formation of SCFAs and altered barrier function of gut epithelium in elderly. These changes associated with ageing, have been described to cause impaired immune functions and may result in a greater susceptibility to disease [ZWIELEHNER et al., 2009].

7.4 RESULTS – BUTYRATE AND BUTYRYL-COA COA-TRANSFERASE GENE

Diet and age are known to influence the production and consumption of SCFAs. Therefore the study done by Hippe *et al.* compared SCFAs in faeces using HPLC-RI and Butyryl-CoA CoA-transferase gene abundance using different sets of degenerated primers and real time quantitative PCR [HIPPE, 2009].

As mentioned above, sets of degenerated primers were used in this study. Some species might not be detected, even if they are present in high numbers, as not from all of the butyrate producing bacteria the Butyryl-CoA CoA-

transferase gene loci is known. Therefore, these primer pairs cover just several butyrate producing bacteria, described by Flint *et al.*, 2006 [HIPPE, 2009].

The highest butyrate concentrations were found in faeces of the mixed diet group; vegetarians had 66.7% lower concentrations and the geriatric group showed 71.43% lower concentrations of butyrate in their faeces [HIPPE, 2009].

Compared to the mixed diet group, in vegetarians significant more Butyryl-CoA CoA-transferase genes (CoATD +191.43%; CTFB +11.86%; PCT+12.5%) were found. In geriatric subjects with one primer set significant more (CoATD +105.7%) and with two primer sets less (CTFB -10% and -20%) abundance of Butyryl-CoA CoA-transferase gene was detected. These results might be due to higher counts of bacteria (12%), *Clostridium* cluster IV (+1.4%) and *Bacteroides* (+7.46%) in the vegetarian group [LISZT *et al.*, 2009], which are known to be butyrate producers. The geriatric subject group had 31% fewer bacteria, while the amounts of *Bacteroides* and *Clostridium* cluster IV were higher compared to mixed diet group. Bifidobacteria were almost the same with 0.8% lower amounts for the geriatric subject group [HIPPE, 2009].

The in situ production of total SCFAs is difficult to determine because more than 95% of SCFAs are rapidly absorbed by the host [MCFALL-NGAI, 2007]. As a result, faecal concentrations of SCFAs are not necessarily representative of concentrations in the colon and can also be affected by intestinal transit time [LEWIS *et al.*, 1997]. Another possibility for fewer concentrations may be the butyrogenic effect [HIPPE, 2009].

Equal to the study discussed before, the elderly group was supplemented with soluble fibre and may resulted in increased Butyryl-CoA CoA-transferase gene abundance and increased butyrate concentrations in their faeces [HIPPE, 2009].

Hippe *et al.* analyzed the same study groups as Zwielehner *et al.*, 2009 and Liszt *et al.*, 2009. The results of Zwielehner *et al.* and Liszt *et al.* can be linked to the results of Hippe *et al.*. Liszt *et al.* reported 12% higher counts of bacterial DNA for vegetarians than for omnivores. The mean proportion of *Clostridium* cluster IV in faeces of vegetarians was $37.2 \pm 17.00\%$ and in omnivores $36.64 \pm 14.22\%$. The average percentage of *Bacteroides* in vegetarians was $28.72 \pm 10.35\%$ and in omnivores $21.26 \pm 8.05\%$. Bifidobacteria were found to make up $1.52 \pm 1.29\%$ in vegetarians and $1.59 \pm 1.73\%$ in omnivores [HIPPE, 2009, LISZT *et al.*, 2009].

8 SUMMARY

The gastrointestinal tract can be regarded as a very complex ecosystem involving interplay between food, host cells and microbes. Microbes inhabiting the human intestine (known as the microbiota) play several significant roles in the digestion of food, generation of short chain fatty acids and prevention of colonization by pathogens. As a balanced and 'healthy' microbiota is important for maintaining human health, strong evidence has emerged of substantial modifications during illness (chronic and degenerative diseases including IBD, Crohn's disease, colon cancer and rheumatoid arthritis) or exposure to threatening experiences, such as antibiotics and chemotherapy.

Although many factors are known to influence the intestinal microbiota, this thesis focuses on diet and SCFAs, ageing and the impact of chemotherapy and antibiotics. This thesis discusses and links the manuscripts by Hippe *et al.* and Lassl *et al.* and the already published article by Zwielehner *et al.*, which can be found in the appendix of the present thesis. However, the main focus of this diploma thesis is on analyzing the microbiota during chemotherapy and antibiotic treatment.

All of these papers used culture independent methods. Abundance of bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster IV and XIVa as well as *Clostridium difficile* was determined using real time quantitative PCR with TaqMan detection. Real time PCR with Sybr-green detection and sets of degenerate primers were used by Hippe *et al.* for Butyryl-CoA CoA-transferase gene abundance measurement. Diversity was assessed applying PCR-DGGE. Furthermore, HPLC-RI was used for detecting faecal concentrations of butyrate.

Ageing and diet are known to alter the composition of gut microbiota. Therefore, faeces of three study populations (industrialized elderly, young omnivores and young vegetarians) were analyzed regarding their butyrate concentration and abundance of Butyryl-CoA CoA-transferase genes by Hippe *et al.*. The Butyryl-CoA CoA-transferase gene is involved in the butyrate production of some bacteria and butyrate, as major energy source for the gut epithelium seems to play an important role for colonic health.

Abundance of Butyryl-CoA CoA-transferase gene increases from omnivores, over elderly to the group of vegetarians, while the average butyrate amount in faeces from HPLC analysis decreases from omnivores, over vegetarians to geriatric population. This study shows that age and diet influences the microbiota and the abundance of butyrate in faeces.

The populations of industrialized elderly and young omnivores were also part of the study by Zwielehner *et al.*. This study aimed at determining ageing-related shifts in diversity and composition of key members of the faecal microbiota. Bacteria, *Bacteroides*, bifidobacteria and *Clostridium* cluster IV were assessed using a combination of molecular methods.

The industrialized elderly harboured significantly higher numbers of *Bacteroides* than controls ($p=0.016$) but contained less bifidobacteria ($p=0.026$) and *Clostridium* cluster IV ($p=0.036$). The elderly showed less total bacteria diversity and less diversity with the *Clostridium* cluster IV and *Bacteroides*. These analyses indicate a less diverse microbiota in elderly.

The main purpose of this study/thesis was to investigate changes within the GI microbiota in response to chemotherapy and antibiotics by assessing diversity and abundance of bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster IV and XIVa and *Clostridium difficile* at four time points in comparison to healthy controls. Therefore, this study aimed to detect shifts in intestinal microbiota, which enable pathogenic *Clostridium difficile* to emerge. Furthermore, the resilience after chemotherapy was investigated.

Patients receiving chemotherapy harboured only $25 \pm 22\%$ of the absolute bacterial load in their faeces compared to healthy controls. After the end of chemotherapeutic cycles the magnitude of microbiota recovers within a few days sometimes even showing a “rebound-effect”. The chemotherapeutical treatment has marginally affected the genera *Bacteroides* and bifidobacteria while the genera *Clostridium* cluster IV and XIVa seem to be more sensitive. While oncology patients harboured significantly more ($p = 0.02$) *Clostridium* cluster IV, controls had more *Clostridium* cluster XIVa. The incidence of *Clostridium difficile* in two subjects was accompanied by a decrease of the genera bifidobacteria and *Clostridium* cluster IV. PCR-DGGE fingerprinting analysis report decreased diversity of bacteria and *Clostridium* cluster XIVa in response to medical treatment and compared to healthy individuals. In conclusion, chemotherapy treatment causes changes in faecal microbiota, which coincides with the development of *Clostridium difficile* infection in some patients.

Altogether, the present thesis shows that diet, ageing and medical treatment such as chemotherapy and antibiotics alter the microbiota of the host organism. As a result, health promoting functions of the microbiota, e. g. SCFAs production is affected. Changes in GI microbiota are linked to impaired immune functions in elderly and immunocompromised subjects and may result in a greater susceptibility to disease and infection. Prebiotic intervention might be a promising concept to modify the microbiota of aged and immunocompromised patients to bring safe health benefits.

9 ZUSAMMENFASSUNG

Mikroorganismen welche den menschlichen Darm besiedeln (Darmmikrobiota) sind eng mit dem Wirtsorganismus verbunden und stellen ein komplexes Ökosystem mit einer Vielzahl an Funktionen dar. Die Unterstützung der Verdauung, Immunmodulation, Synthese von Vitaminen und Produktion von Buttersäure zur Energieversorgung der Darmepithelien sind nur einige der Aufgaben, welche das Mikrobiota für den Wirtsorganismus erfüllen. Eine ausgeglichene und ‚gesunde‘ Mikrobiota ist zusätzlich notwendig um das Wachstum von pathogenen Mikroorganismen, wie in etwa *Clostridium difficile* im Zaum zu halten und ist deshalb für die Gesunderhaltung des Menschen von enormer Bedeutung. In der Vergangenheit wurden auch Zusammenhänge zwischen Erkrankungen (chronisch entzündliche Darmerkrankung, Morbus Crohn, Darmkrebs, Rheumatoide Arthritis, Adipositas, ect.) und einer veränderten Darmmikrobiota gefunden. Weiters haben Medikamente, wie Zytostatika im Rahmen einer Chemotherapie und Antibiotika einen merklichen Einfluss auf die Zusammensetzung des Mikrobiota.

Die vorliegende Diplomarbeit beschäftigt sich mit den Zusammenhängen zwischen der Darmmikrobiota und dem Einfluss der Ernährung und Butyrat, dem Einfluss des Alterns und dem Einfluss von Chemotherapie und Antibiotika. Diese Arbeit gibt einen Überblick über die im Anhang zu findenden Manuskripte von Hippe *et al.* und Lassl *et al.*, sowie dem veröffentlichten Artikel von Zwieler *et al.*. Das Hauptaugenmerk dieser Arbeit liegt jedoch in der Analyse des menschlichen Mikrobiota während Chemo- und Antibiotikatherapie.

Alle Analysen wurden mit Hilfe von Kultur-unabhängigen Methoden durchgeführt. Das Vorhandensein von Bakterien, *Bacteroides*, bifidobakterien, *Clostridium* cluster IV, XIVa und *Clostridium difficile* im Stuhl wurde mit real time PCR (TaqMan-Detektion) analysiert. Diversitätsanalysen wurden mit PCR-DGGE durchgeführt. Einzig das Vorhandensein des Butyryl-CoA CoA-transferase Gens wurde anhand eines Sybr-green Systems für real time PCR

nachgewiesen, während die Buttersäure-konzentrationen im Stuhl mit HPCL-RI gemessen wurden.

Hippe *et al.* untersucht Stuhl von drei verschiedenen Populationen (Geriatriker, junge Mischköstler und junge Vegetarier) hinsichtlich Buttersäure-konzentrationen und dem Vorliegen des Butyryl-CoA CoA-transferase Gens. Die Butyryl-CoA CoA-transferase spielt eine Rolle bei der bakteriellen Produktion von Buttersäure, welche als Faktor für die Gesunderhaltung des Darms gilt.

Das Butyryl-CoA CoA-transferase Gen wurde am häufigsten in der Gruppe der Vegetarier gefunden, gefolgt von Geriatrikern und Mischköstlern. Im Gegensatz dazu wurde die höchste Buttersäure-konzentration im Stuhl von Mischköstlern, gefolgt von Vegetariern und Geriatrikern festgestellt. Diese Ergebnisse zeigen eine vermehrte Ansiedelung von Buttersäure-produzierenden Bakterien im Darm von Vegetariern. Das vermehrte Vorkommen von Buttersäure im Darmlumen könnte laut Hippe *et al.* die Vermehrung von Bakterien unterstützen, welche Butyrat als Substrat verwenden und deshalb zu verminderten Buttersäure-konzentrationen im Stuhl führen.

Weiters wurde die Gruppe der Geriatriker mit jener der jungen Mischköstler von Zwielehner *et al.* untersucht, um alterungs-relevante Veränderungen des Mikrobiota zu bestimmen. Zu diesem Zwecke wurden das Vorkommen und die Diversität von Bakterien, *Bacteroides*, bifidobakterien und *Clostridium* cluster IV analysiert.

Zwielehner *et al.* konnte signifikant mehr *Bacteroides* ($p=0.016$), jedoch weniger bifidobakterien ($p=0.026$) und *Clostridium* cluster IV ($p=0.036$) im Stuhl von Geriatrikern im Vergleich zu Kontrollpersonen nachweisen. Ältere Personen zeigten außerdem eine verringerte Diversität hinsichtlich der Gruppen *Clostridium* cluster IV und *Bacteroides*. Diese Resultate zeigen ein verändertes und weniger diverses Mikrobiota im Stuhl älterer Personen.

Hauptsächlich fokussierte diese Arbeit jedoch auf Veränderungen und Verschiebungen innerhalb des Mikrobiota aufgrund von Chemotherapie und Antibiotika. Daher wurden Diversität und Vorhandensein von Bakterien, *Bacteroides*, bifidobakterien, *Clostridium* cluster IV und XIVa sowie *Clostridium difficile* zu vier verschiedenen Zeitpunkten der Therapie untersucht. Die wichtigsten Fragestellungen lauteten in diesem Zusammenhang: Ist das Mikrobiota nach Chemotherapie fähig sich zu erholen bzw. seine ursprüngliche Zusammensetzung wieder zu erlangen? Und kann im Laufe der Therapie das Heranwachsen von *Clostridium difficile* beobachtet werden?

Bei Chemotherapie-patienten konnten im Vergleich zu Kontrollpersonen nur $25 \pm 22\%$ an Bakterien im Stuhl nachgewiesen werden. Die Behandlung mit Zytostatika zeigte wenige Veränderungen hinsichtlich *Bacteroides* und Bifidobakterien, während *Clostridium* cluster IV und XIVa merkliche Veränderungen zeigten. Interessanterweise konnten bei Chemotherapie-patienten signifikant mehr ($p=0.02$) *Clostridium* cluster IV nachgewiesen werden, während *Clostridium* cluster XIVa in größerer Anzahl im Stuhl von Kontrollpersonen zu finden war. Nach Chemotherapie konnte sich das Mikrobiota, nach einem gesamten Absinken, häufig wieder auf Ausgangsniveau und auch darüber hinaus erholen. Das Vorkommen von *Clostridium difficile* konnte bei zwei Chemotherapie-patienten beobachtet werden. DGGE-Analysen des Mikrobiota von Chemotherapie-patienten konnte eine verringerte Diversität bei Bakterien und *Clostridium* cluster XIVa feststellen.

Abschließend konnten diese Ergebnisse zeigen dass Alter, Ernährung und der Einfluss von Medikamenten wie Zytostatika und Antibiotika einen erheblichen Einfluss auf Zusammensetzung und Diversität des Darmmikrobiota haben. Folglich kann eine Vielzahl an gesundheitsfördernden Funktionen des Mikrobiota beeinträchtigt sein. Inwieweit eine prebiotische Supplementierung die Wiederherstellung bzw. Erhaltung einer ‚gesunden‘ Mikrobiota bei älteren und immunsupprimierten Patienten beeinflussen kann, ist Gegenstand der Forschung in nächster Zukunft.

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11 PUBLISHED RESEARCH DATA

11.1 MANUSCRIPT – “CHANGES IN HUMAN FAECAL MICROBIOTA DUE TO CHEMOTHERAPY ANALYZED BY TAQMAN-PCR AND PCR-DGGE FINGERPRINTING”

CHANGES IN HUMAN FAECAL MICROBIOTA DUE TO CHEMOTHERAPY ANALYZED BY TAQMAN-PCR AND PCR-DGGE FINGERPRINTING

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ABSTRACT

Chemotherapy and antibiotics have been shown to disturb the host's protective gastrointestinal microbiota, thereby increasing susceptibility to infection. We investigated consequences of chemotherapy $\pm$ antibiotics in cancer treatment on abundance and diversity of dominant faecal microbiota.

Faeces of 11 ambulant patients receiving chemotherapy $\pm$ accompanying antibiotics were analyzed before and after 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 *Clostridium difficile* with TaqMan-qPCR. Furthermore, we assessed diversity of bacteria, *Clostridium* clusters IV and XIVa at the four sampling points using denaturing gradient gel electrophoresis (DGGE) fingerprinting.

Abundance of faecal microbiota decreases significantly after administration of chemotherapeutics as well as in comparison to healthy controls. The abundance of microbiota recovers within a few days sometimes even showing a "rebound-effect". Treatment led to a significant increase of relative abundance of *Clostridium* cluster IV and a decrease of *Clostridium* cluster XIVa. *Bacteroides* and bifidobacteria were marginally affected. In addition, diversity of bacteria and *Clostridium* cluster XIVa decreased in response to treatment and in comparison to healthy individuals. In two out of eleven patients *Clostridium difficile* emerged.

Despite high individual variations these results suggest that abundance and diversity of gastrointestinal microbiota, especially *Clostridium* cluster XIVa following treatment facilitate growth of emerging pathogens, such as *Clostridium difficile*.

INTRODUCTION

The intestinal ecosystem constitutes the microbiota that can be "pictured as a microbial organ placed within a host organism". This ecosystem involves a dynamic interplay between food, host cells and microbes [TURRONI et al., 2008]. Recent studies reveal that major mammalian metabolic processes are

under symbiotic homeostatic control [MARTIN et al., 2009]. The microbiota is significantly involved in the digestion of food, energy regulation, generation of short chain fatty acids (SCFAs), vitamin synthesis, prevention of pathogen colonization and protection against cell injury [BACKHED et al., 2005, DE LA COCHETIERE et al., 2005, ECKBURG et al., 2005, TLASKALOVA-HOGENOVA et al., 2004, TURNBAUGH et al., 2009].

The faecal microbiota is dominated by the *Clostridium coccoides* group - *Clostridium* cluster XIVa (lately reclassified by Liu et al. as *Blautia coccoides*) the *Clostridium leptum* group - *Clostridium* cluster IV and *Bacteroides* group [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]. Abundance and diversity of dominant bacteria are affected by ageing and diet [LISZT et al., 2009, ZWIELEHNER et al., 2009].

Innate immunity, mechanical mucosal barrier and colonization resistance prevent the invasion of endogenous bacteria from oral cavity and the gastrointestinal tract [VAN VLIET et al., 2009]. Changes of *Firmicutes* and *Bacterioidetes* and weakened barrier function is currently discussed for complex diseases such as metabolic syndrome and atopy [LEY et al., 2005, SUZUKI et al., 2008].

Chemotherapy and the use of antibiotics damage the mucosal cells of the gastrointestinal tract and disrupt the ecological balance of the microbiota, allowing pathogens such as *Clostridium difficile* to emerge [GUARNER et al., 2003, NYHLEN et al., 2002]. *Clostridium difficile* is thought to be the causative agent in up to 20% of antibiotic-associated diarrhoea (AAD) patients [KONING et al., 2008].

Approximately 40% of patients receiving standard dose chemotherapy and 100% of patients receiving high dose chemotherapy and stem cell or bone marrow transplantation have been described to 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 dedicated to changes in the microbiota composition. Because of these changes, absorption and other intestinal functions of the microbiota may also be altered [GIBSON et al., 2006].

Whereas consequences of disturbed GI microbiota are generally accepted, changes of the microbial balance which allow emergence of pathogens need to be better defined.

We analyzed the course of faecal microbiota of patients receiving cancer chemotherapy $\pm$ antibiotics in comparison to healthy control individuals. We focused on abundance and diversity of dominant bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster IV, *Clostridium* cluster XIVa and *Clostridium difficile* using culture-independent methods. Changes in populations were analyzed for the ability to return to its original composition (resilience) after chemotherapy and the emergence of *Clostridium difficile*.

MATERIAL AND METHODS

Study participants and study design

Eleven subjects receiving chemotherapy $\pm$ 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 before, during or after the onset of treatment within two weeks. The term “after chemotherapy” is used in the following sections for sampling points after chemotherapeutic intervention (1 – 7 days after). Two out of nine patients had never received any chemotherapy before, while the others had a longer history of chemotherapy. Stool samples of healthy individuals were collected once.

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 for at least three months before the collection of faecal samples. One subject receiving chemotherapy additionally suffered from rheumatism while another subject suffered from diabetes mellitus type II, hypertension and obesity. Study populations were gender balanced, with 55% females in both, oncology patients and healthy controls.

Furthermore, patients' medical records reported chemotherapeutic and antimicrobial treatment. All subjects gave written informed consent. The study was approved by the ethics committee of Vienna.

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 (QIA-GEN) following the manufacturer's protocol. Finally we stored the DNA at -20°C until the analysis was conducted.

Type strains

We used type strains, knowing to be a 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, *Lactobacillus 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 (Fig. 3).

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. We picked forty clones, amplified with primer pair T7/Sp6 (Promega) and checked fragment length on 2% agarose gel before clone inserts were sequenced by 'DNA confidence' (Vienna). Finally, nucleotide sequences were corrected for primer and vector sequences in CodonCodeAligner (www.codoncode.com) and taxonomically identified using 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].

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 2. The PCR reaction mixture consisted of ready-to-use mastermix (Promega) with 1.5 mM MgCl₂, 500 nmol/L of both primers and 2 µl of template DNA. When amplifying faecal samples, bovine serum albumin (Fermentas) was added to a final concentration of 400 µg/ml. We used a Robocycler (Stratagene) for all amplifications.

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 using a gradientmixer (Hoefer SG 30) and a peristaltic pump. 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 gradient-variations within one gel.

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 1) 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. Briefly, 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 in a final volume of 10µl see table 1) and 10ng of bacterial DNA. Amplification programs included an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation step at 95 °C for 30 s, primer annealing at 55 °C (*bacteria*, *Clostridium* cluster IV), 56 °C (*Clostridium* cluster XIVa), 58 °C (*Clostridium 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 *Clostridium 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 *Clostridium difficile*, using the nanodrop method and calculated DNA copies/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].

Sensitivity of PCR reactions was determined with stepwise dilutions of standard curve DNA. The specificity was confirmed using non-target DNA.

Statistical analysis

Statistical evaluation of differences between groups (chemotherapy and control) and changes within the chemotherapy group (time points before chemotherapy, time points 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-ranked-test. P values below 0.05 were considered statistically significant.

We analyzed PCR-DGGE bands in GelComparII (www.applied-maths.com) and compared band tables 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. Clustering was performed applying Dice coefficient and UPMGA dendrograms. 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 as well as whole grain products and alcohol several times a week compared to patients receiving chemotherapy (not significant).

Chemotherapeutic treatment $\pm$ antibiotics decreases absolute bacterial numbers in comparison to healthy controls

To investigate whether chemotherapy $\pm$ antibiotics change the human faecal microbiota composition over time and in contrast to healthy individuals, we investigated absolute numbers and relative percentages of bacterial subgroups. The ambulant patients receiving chemotherapy harboured only $25 \pm 22\%$ of the absolute bacterial load in their faeces compared to healthy controls. This overall

decrease affected *Bacteroides*, bifidobacteria, *Clostridium* cluster IV and XIVa. Despite high inter- and intraindividual variations among samples, the differences in absolute numbers of bacteria ($p = 0.02$), *Bacteroides* ($p=0.01$), bifidobacteria ($p=0.001$) and *Clostridium* cluster XIVa ($p=0.001$) were statistically significant. Furthermore, patients receiving chemotherapy had less absolute numbers of *Clostridium* cluster IV (not significant). The decrease of all bacterial subgroups within 3 – 6 days after chemotherapeutic intervention was in some cases followed by a rebound of bacterial abundance within 5 – 9 days (Figure 1). One subject showed a drastic decline of all analyzed bacteria, following treatment due to blood stem cell transplantation.

***Clostridium* cluster IV and XIVa show great alterations due to chemotherapeutic intervention**

The mean proportion of *Bacteroides* in stool samples was $27 \pm 12\%$ in chemotherapy patients and $23 \pm 12\%$ in healthy individuals. The mean percentage of bifidobacteria in patients was $1.17 \pm 1\%$ and $1\% \pm 0.7\%$ in controls. Patients harboured $16 \pm 11\%$ of *Clostridium* cluster IV and $20 \pm 14\%$ of *Clostridium* cluster XIVa, while controls harboured $10 \pm 7\%$ and $34 \pm 22\%$ of clostridial clusters IV and XIVa (Figure 2). Subgroups were quantified as percentage of total bacterial DNA.

A difference between patients receiving chemotherapy and control group concerning the subgroups *Clostridium* cluster IV and XIVa was observed. While patients receiving chemotherapy harboured significantly more ($p = 0.02$) *Clostridium* cluster IV, controls had more *Clostridium* cluster XIVa (Figure 4A).

Decreased levels of bifidobacteria and *Clostridium* cluster XIVa and increased levels of *Clostridium* cluster IV after chemotherapeutic intervention

The average amount of bifidobacteria before chemotherapy was $1.6 \pm 2\%$ in comparison to $0.8 \pm 1.4\%$ after chemotherapy, but not significant ($p=0.06$). Furthermore, *Clostridium* cluster XIVa showed a decrease from $23 \pm 17\%$ to $15 \pm 9\%$ comparing time points before Chemotherapy and time points after

Chemotherapy. The mean percentage of *Clostridium* cluster IV at time points before chemotherapy was 15 ± 12 %, compared to time points after chemotherapeutic intervention (defined in Material and Methods) with 18 ± 14 % of all analyzed bacteria. No difference concerning the subgroup *Bacteroides* was observed (Figure 4C).

***Clostridium difficile* colonization following chemotherapy**

In 18% of patients receiving chemotherapy *Clostridium difficile* was detected. One Patient harboured *Clostridium difficile* at all time points investigated (of 0.4 ± 0.7 % of total DNA, showing the highest level of 1.22% at sampling point 3, as highlighted in Figure 2), after chemotherapeutic and antibiotic treatment. In one patient *Clostridium difficile* emerged at time points 3 (3.90% of total bacterial DNA, Figure 2).

Higher bacterial and clostridial XIVa diversity in healthy controls

DGGE fingerprinting analyses of bacteria, *Clostridium* cluster IV and *Clostridium* cluster XIVa subgroups show highly diverse datasets in individuals indicating a pronounced individual uniqueness of faecal microbiota. The mean numbers of bacterial bands per patient receiving chemotherapy were 21.8 ± 5 , of *clostridium* cluster IV 9 ± 5 bands and of *clostridium* cluster XIVa 14.9 ± 7 bands. 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 amount of bands as well as Shannon and Simpson diversity indices show a higher diversity of bacteria and *Clostridium* cluster XIVa in healthy controls (Figure 5). In contrast to bacterial and clostridial cluster XIVa fingerprints, clostridial cluster IV DGGE fingerprints show higher diversity indices in patients receiving chemotherapy. After chemotherapy a decrease of the bacterial and clostridial cluster IV diversity, but a slightly higher clostridial cluster XIVa diversity was seen compared to time points before chemotherapy (Figure 5).

The dataset was subjected to principal component analysis (PCA). PCA analysis extracts underlying components of samples according to their variance. PCA of *Clostridium* cluster IV fingerprints indicates separation of time points

after chemotherapy (O), before chemotherapy (T) and control individuals (C), Figure 5. PCA analysis thus shows distinctive clustering of O and T samples. Bands 178.16, 169.81 and others are characteristic for O samples. Bands 207.44, 210.53, 208.17, 302.36, 204.66 and 299.79 are characteristic for samples T and responsible for the separation of samples T and O.

DISCUSSION

Chemotherapy and antibiotics are essential standard therapies in malignant diseases but severe side-effects such as mucositis, diarrhoe or constipation are often life-threatening [STRINGER et al., 2007]. This treatment severely disturbs the host microbiota, host mucosal protection [CROSWELL et al., 2009], thereby increasing the risk of infections [VAN VLIET et al., 2009]. Overgrowth of species with potential pathogenicity such as toxigenic *Clostridium difficile* and inflammatory complications are among the most-encountered serious complications of chemotherapy and antibiotic treatment in cancer patients [GUARNER et al., 2003, VAN VLIET et al., 2009].

In this study, we investigated how the use of cancer chemotherapy $\pm$ antibiotic treatment perturbs the faecal microbial ecosystem over the course of the therapy. The core questions were if the microbiota is able to return to its original profile after chemotherapeutic and/or antibiotic intervention and if *Clostridium difficile* could be detected more often following therapy. We compared abundance (qPCR) and diversity (PCR-DGGE) of bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster IV, *Clostridium* cluster XIVa and *Clostridium difficile* in faecal microbiota.

Faecal microbial communities are composed of autochthonous gut members and by transient bacteria, consequently analysis of faecal samples may overestimate the actual composition of the GIT microbiota. However, faecal samples reflect shifts in populations [TURRONI et al., 2008].

We used type strains *Bacteroides thetaiotaomicron*^T, *Bifidobacterium longum* ssp. *longum*^T and *Clostridium difficile* as well as cloned sequences for quantification. However, a mixture of different strains for qPCR standards might be more accurate because some species might be preferentially amplified.

Therefore, qPCR results should be considered as semiquantitative and results focus mainly on comparative quantification.

Diversity was assessed using the PCR-DGGE method. Several species possess more than one 16S rRNA operon such as *E. coli* IMBH 252/07 who forms 4 DGGE bands (FIG. 3). The limitations of DGGE in microbial analysis have been previously explored [MUYZER et al., 1998]. Nevertheless, DGGE analysis yields substantial information from very complex microbial communities [MUYZER et al., 1998].

The group of patients receiving chemotherapy involved patients with and without antibiotic therapy. Many study participants had a longer history of different chemotherapeutic regimens. Two individuals had never received any cancer therapy before. Types of cancer chemotherapeutic therapy was variable as described in Material and Methods. Patient who did not receive any antibiotics showed similar alteration than those under antimicrobial therapy. Van Vliet *et al.*, 2009 tested the effect of chemotherapy in vitro and showed a direct bacteriostatic effect of chemotherapeutics on bacterial growth. Despite this variability in clinical protocols results from changes in microbiota due to chemotherapeutic administration is remarkably homogenous.

The majority previous studies on the effect of chemotherapy on human fecal microbiota used culture techniques [NYHLEN et al., 2002, STRINGER et al., 2007]. Many studies focused on the colonization with pathogenic bacteria [SCHALK et al., 2009, VAN VLIET et al., 2009] in patients with cancer and chemotherapy-induced diarrhea [ABD EL-ATTI et al., 2009, STRINGER et al., 2007].

The mean proportion of *Bacteroides* in faecal samples was $27 \pm 12\%$ in chemotherapy patients and $23 \pm 12\%$ in healthy individuals. These results are not in agreement with the results of Nyhlén *et al.*, 2007 [NYHLEN et al., 2002] who showed increased counts of *Bacteroides* spp. of samples obtained during chemotherapy. Discrepancies in changes of the *Bacteroides* abundance might be due to differences in detection techniques, as Nyhlén *et al.* used culture methods. Nyhlén *et al.*, 2007 also reported significantly increased counts of

yeast in patients, making it a focus for further research in immunocompromised patients.

Our results show 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 abundances of bacteria and determined bacterial subgroups, PCR-DGGE fingerprints indicate lower diversity of bacteria and *Clostridium* cluster XIVa in patients receiving chemotherapy. Faecal microbiota decreases after administration of chemotherapeutics. After the end of chemotherapeutic cycles the magnitude of microbiota recovers within a few days sometimes even showing a “rebound-effect”, supporting the hypothesis that the microbiota is able to recover after chemotherapeutic intervention. Relative numbers of *Clostridium* cluster IV and XIVa show great alterations due to chemotherapeutical interventions, while the genera *Bacteroides* and bifidobacteria seem to be less affected. Interestingly, patients receiving chemotherapeutics harboured significantly more *Clostridium* cluster IV but at lower diversity than controls. This suggests that different clusters of Clostridia contain species that are particularly vulnerable or even take advantage of disruption of community composition. The clostridial clusters are known to harbour a great diversity of phylogenetically loosely related bacteria. Emergence of *Clostridium difficile* may reflect changes in the community composition within the Clostridia.

Further research needs to evaluate whether the observed changes in microbiota also play a role in the maintenance of the mucosal barrier functions, infection and inflammation [VAN VLIET *et al.*, 2009].

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TABLES

TABLE 1. Primers and TaqMan-probes targeting 16S rRNA coding regions of faecal bacteria for absolute and relative quantification used in this study.

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

FIGURES

FIG. 1. 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), *Clostridium 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 sampling.

FIG. 2. Percentage of *Bacteroides* (Bac), bifidobacteria (Bif), *Clostridium* cluster IV (Clept) and XIVa (Ccocc), *Clostridium difficile* (Cdiff) and unidentified bacteria of analyzed bacteria quantified by TaqMan-qPCR.

FIG. 3. Changes of PCR-DGGE fingerprinting of 16S rRNA coding regions of dominant bacteria after chemotherapeutic treatment. Bands that get stronger or nearly disappear are indicated with arrows. SL: standardlane, A, B, C and D: ON001 receiving chemotherapy, E: healthy control.

FIG. 4. (A) Relative amount of faecal bacteria in patients receiving chemotherapy $\pm$ antibiotics (ON) in comparison to healthy individuals (C). Percentage of *Bacteroides* (bac), bifidobacteria (bif), *Clostridium* cluster IV (Clept) and cluster XIVa (Ccocc) in relation to detected bacteria. (B) Comparison of samplings during chemotherapeutic treatment with samplings without chemotherapeutic cycles. Percentage of *Bacteroides* (bac), bifidobacteria (bif), *Clostridium* cluster IV (Clept) and cluster XIVa (Ccocc) related to all analyzed bacteria. (C) Time points before Chemotherapy in comparison to time points after Chemotherapy.

FIG. 5. Simpson and Shannon diversity indices derived from PCR-DGGE bandpattern of 16S rRNA coding regions of (A, B) bacteria, (C, D) *Clostridium* cluster IV and (E, F) *Clostridium* cluster XIVa. ON: Oncology patients receiving chemotherapy, C: healthy controls, ChT: sampling points during ChT, noChT: sampling points without chemotherapy, beforeChT: samplings before chemotherapy, afterChT: samplings after chemotherapy.

FIG. 6. Principal component analysis (PCA) of *Clostridium* cluster IV DGGE fingerprints of 16S rRNA coding regions of dominant bacteria in faecal samples of patient receiving chemotherapy and healthy controls. Grey circles indicate time points after chemotherapy (O), black oval indicates timepoints before chemotherapy (T) and grey thin oval indicates the location of control samples (C) in the plot.

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11.2 MANUSCRIPT – “CONSEQUENCES OF DIET AND AGEING FOR INDIVIDUAL SCFAS AND FAECAL MICROBIOTA”

CONSEQUENCES OF DIET AND AGING FOR INDIVIDUAL SCFAS AND FAECAL MICROBIOTA

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ABSTRACT

Short chain fatty acids (SCFAs) are end- and intermediate products of microbial breakdown of polysaccharides in the human gut. Especially butyric acid serves as major energy source for the gut epithelium and regulates inflammatory pathways and gene expression.

The influence of microbiota and diet on inter individual differences in metabolites, particularly SCFAs, and colonic health is poorly understood. The aim of the study was to investigate whether the individual composition of microbiota affected by diet or age results in a specific difference in butyrate production. SCFAs were measured in faecal samples of elderly ($n = 17$; age = 86 ± 8), young omnivores ($n = 17$; age = 24 ± 2.5) and young vegetarians ($n = 16$; age = 26 ± 5) using HPLC-RI. Abundance of the Butyryl-CoA CoA-transferase gene was analysed with gene specific real-time PCR applying three degenerate primer sets. Abundance of Archaea was estimated with a TaqMan real-time PCR system.

Results:

Butyric acid levels in faeces ranged between below detection limit ($>0.0024 \mu\text{mol}^{-9}$) up to $0.095 \mu\text{mol}^{-9}$ faeces. Vegetarians showed significantly lower butyric acid levels (68 %, $p=0.06$) compared to omnivores. Geriatric subject group showed lower levels of butyric acid compared to young omnivores, but not significant.

The main short chain fatty acids found in faecal samples of most subjects were acetic acid, tartaric acid, lactic acid and propionic acid.

Butyryl-CoA CoA transferase gene levels showed higher abundances (3 times more, $p=0.0001$) in vegetarians than in omnivores. The average abundance of the Butyryl-CoA CoA transferase gene was $5.26 \times 10^8 \pm 2.38 \times 10^8$ copies $^{-\mu l}$ in omnivores, $5.26 \times 10^8 \pm 2.38 \times 10^8$ copies $^{-\mu l}$ in elderly and $7.46 \times 10^8 \pm 2.29 \times 10^8$ copies $^{-\mu l}$ in vegetarians.

Archaea were nine times more abundant in omnivores, and in elderly (2.8 times more often) than in vegetarians.

Conclusion: We show that age and diet influence the microbiota and the butyric acid abundance in faeces. Vegetarian diet favours growth of microbiota including SCFA producing bacteria in the gut lumen.

INTRODUCTION

The composition of GI microbiota is highly distinct between individuals [DETHLEFSEN et al., 2006] and different bacteria interact and influence each other in abundance and relation [NEISH, 2009]. These interactions and other factors influencing the intestinal microbiota and its establishment are related to food sources including delivery and nutrition [DUNCAN et al., 2007], life style factors, medical treatment and age [GUIGOZ et al., 2008] as well as genetic factors (Khachatryan et al. 2008).

An important metabolic process of anaerobic colonic bacteria is their production of SCFAs. Strains that produce particularly high amounts of SCFAs from the breakdown of dietary fibre have been identified in the *Bacteroides* genus and - amongst others- the *Roseburia*, *Eubacteria* and *Butyrivibrio* geni. Cell culture studies have indicated that the presence of butyrate at physiological concentrations enhances the growth of normal enterocytes and inhibits that of malignant ones [HINNEBUSCH et al., 2002]. Butyrate may have an anticarcinogenic and anti-inflammatory potential, affect the intestinal barrier and play a role in satiety and oxidative stress [HAMER et al., 2008]. Butyrate induces apoptosis in colorectal tumour cell lines, reduces metastasis, and protects from genotoxic carcinogens by enhancing expression of phase II detoxification enzymes [POOL-ZOBEL et al., 2007]. Acetate and butyrate promote methane production, while propionate formation is considered as a

competitive pathway for hydrogen use (Moss et al. 2000). Organic acid degradation depends on the presence of consortia of acetogenic bacteria and methanogenic Archaea (Stams et al. 2005). The majority of Archaea in the human GI-tract are related to *Methanobrevibacter smithii* (Dethlefsen, L et al. 2006).

Because of the rather low consumption of fermentable dietary fiber in today's Central European diet the bacterial diversity in the lumen may vary, and with that the butyric acid concentrations. Our group has recently shown differences between the bacterial diversity in Vegetarians and Elderly compared to omnivores (Zwielehner et al. 2009; Liszt et al. 2009). Based on these previous results we investigate whether the individual composition of the microbiota results in a specific difference in butyric acid production and abundance.

Faecal samples of young healthy individuals with omnivore or vegetarian diets as well as institutionalized elderly were analysed with HPLC-RI and TaqMan real-time PCR to quantify SCFAs and the abundance of the Butyryl-CoA CoA-transferase gene.

EXPERIMENTAL METHODS

Study subjects

DNA samples and subject group range were used from prior research carried out by our working group as published (Zwielehner et al. 2009, Liszt et al. 2009).

The geriatric group (Elderly) is formed by 17 institutionalized subjects aged 86 ± 8 years BMI 21.75 ± 5.08 , from a geriatric department in Vienna. The vegetarian diet group (Vegetarian) is formed by 16 young healthy subjects with a vegetarian or vegan diet, aged 26 ± 5 years with BMI 21.02 ± 2.71 . Seventeen young healthy subjects aged 24 ± 2.5 years, BMI 22.68 ± 3.41 with a Central European diet form 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 are gender balanced. Only non-pregnant probands with no diagnosed gastro-intestinal disease and no antibiotic or chemotherapeutic treatment up to three months prior to sampling were included in the study. All probands agreed to participate in the study and gave their informed consent. Approval was obtained from the Ethics committee of the city of Vienna.

Faecal sample collection

Faecal samples were collected from each participant individually and directly stored at -18°C. Faecal aliquots were taken for DNA extraction and SCFA analysis with HPLC.

Quantification of functional genes and archaeal 16S rRNA gene with real-time PCR

Human colonic butyrate producers use Butyrylcoenzyme A CoA transferase [CHARRIER et al.] for the last step of butyrate formation. The CoA-transferase gene of the colonic bacterium *Roseburia sp. A2-183* is similar to acetyl-CoA hydrolase as well as 4-hydroxybutyrate CoA-transferase sequences. Primer pair CoACT was described to target 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) and e.g. for PCT primer pair: *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 the functional genes with three degenerate primer sets as described in table 1 (Charrier et al. 2006). 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 real-time PCR as listed in table 1. Conditions for real-time PCR with a 4 pmol primer and probe concentration were: 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 20s at 72°C, a final elongation for 1min at 72°C.

The different primer sets were applied to the stool samples and the PCR products quantified using the nano drop method. These PCR products served as standards for absolute and relative quantitation in real-time PCR. A relative quantitation was done for Archaea rRNA gene abundances to assess a possible correlation with Butyryl CoA CoA transferase gene abundances.

Based target Organism/Gene	Primer pairs or probe	Sequence (5'-3')	Reference
CoA Transferase based on <i>Roseburia</i> sp. A2-183 (AY796317)	CoATD for	AAGGATCTCGGIRTICAYWSIGARATG	C. Charrier et al. 2006
	CoATD rev	GAGGTCGTCICKRAAITYIGGRTGNCG	
CoA Transferase based on <i>C. acetobutylicum</i> (P23673)	CTFB for	GTAAACTTIGGIRTIGGIYTNCCNAC	C. Charrier et al. 2006
	CTFB rev	AACAGTAACATCIAYRTGICCNCCNC	
CoA Transferase based on <i>C. perfringens</i> (NP_561012)	PCT for	GTAGGATTARRIACITWYRTIGAYCC	C. Charrier et al. 2006
	PCT rev	TCCACCACCATCRTARSARTCRAAYTG	
Archaea	ARC787F F	ATTAG ATACC CSBGT AGTCC	Y.Yu et al. 2005
	ARC915F		
	TaqMan	AGGAA TTGGC GGGGG AGCAC	
	ARC1059R R	GCCAT GCACC WCCTC	

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

SCFA in faeces

SCFA concentrations in faecal specimens were measured according to the method of Huel-Mel Chen et al.1998. The faeces were collected and stored frozen. After faeces were thawed, 3g were homogenised in a Stomacher Lab-Blender 400 for 2 minutes in 20 ml of 0.15 mmol/L H₂SO₄ in bidest-water. The homogenate was centrifuged at 6000 x g at 2 °C for 60 min. The supernatant

fluid was then twice filtered through a micro filter syringe with 0.22 μm . The filtrate received no further treatment and was directly injected into the HPLC.

SCFA	Retention time (min.)	detection Limit ($\mu\text{mol/g}$)
Acetic acid	3.04	0.0010
Tartaric acid	7.93	0.0016
Lactic acid	6.86	0.0026
Formic acid	7.52	
Fumaric acid	8.29	
Propionic acid	9.70	0.0043
Butyric acid	11.98	0.0024

Table 2: Retention times and limits under detection of measured short chain fatty acids.

The standard calibration was determined from the average of five 0.95 mg/L injections for each standard acid solution as displayed in table 2. The HPLC apparatus (Dionex 3000) consisted of a mobilephase reservoir; a Dionex ICS 3000 sp pump; an automatic sample injector, Dionex AS; a MetaCarb 67H organic acid chromatographic column, 300mm; a temperature control module; a refractive index detector; a chromatography control station and a system interface module.

The mobile phase a 0.005 mMol solution of “ultra pure” sulphuric acid (VWR) in Milli-Q purified water (pH 3.1, background conductance <100 MS), was filtered through a 0.2 μm (Herba chemosan) membrane and degassed before use. Separation of SCFAs [HINNEBUSCH et al.] 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 ultra pure sulphuric acid at a flow rate of 0.8 ml/min for 2 h.

Analysed data and statistical interpretation

Elementary statistical analysis (mean, standard deviation and coefficient of variation (F-test) was applied to the data (OriginPro8). 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 Rotorgene 3000 and transferred to Excel. Food frequency data collected with a food frequency questionnaire was analysed using the Chi-square approximation as implemented in SPSS15. All tests were double sided, $p > 0.05$ was considered as significant.

RESULTS

Dietary analysis

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

Quantification of the Butyryl-CoA CoA-transferase gene

Applying the CoATD primer set, Vegetarians ($7.46 \times 10^8 \pm 2.29 \times 10^8$ copies $^{-\mu l}$) had the highest abundance of the Butyryl-CoA CoA-transferase gene among all specimens. The abundance was significantly higher than for Omnivores ($5.26 \times 10^8 \pm 2.38 \times 10^8$ copies $^{-\mu l}$; $p = 0.0001$) and Elderly ($p = 0.01$). The abundance of this functional gene in Elderly ($5.26 \times 10^8 \pm 2.38 \times 10^8$ copies $^{-\mu l}$) showed the highest variation, being significantly higher than those found in Omnivores ($p = 0.0007$) but lower than in Vegetarians.

Quantification with a CTFB primer yielded a gene abundance of $4.12 \times 10^8 \pm 1.81 \times 10^8$ copies $^{-\mu l}$ for Omnivores, $4.6 \times 10^8 \pm 2.51 \times 10^8$ copies $^{-\mu l}$ for Vegetarians and $3.7 \times 10^8 \pm 1.89 \times 10^8$ copies $^{-\mu l}$ for Elderly. Applying the PCT primer pair, abundances of $8.61 \times 10^8 \pm 3.12 \times 10^8$ copies $^{-\mu l}$ for Omnivores, $8.53 \times 10^8 \pm 5.28 \times 10^8$ copies $^{-\mu l}$ for Vegetarians and $6.55 \times 10^8 \pm 4.73 \times 10^8$ copies $^{-\mu l}$

for Elderly were found. There were no significant differences, but a trend for lower abundances in Elderly and slightly higher abundances in Vegetarians.

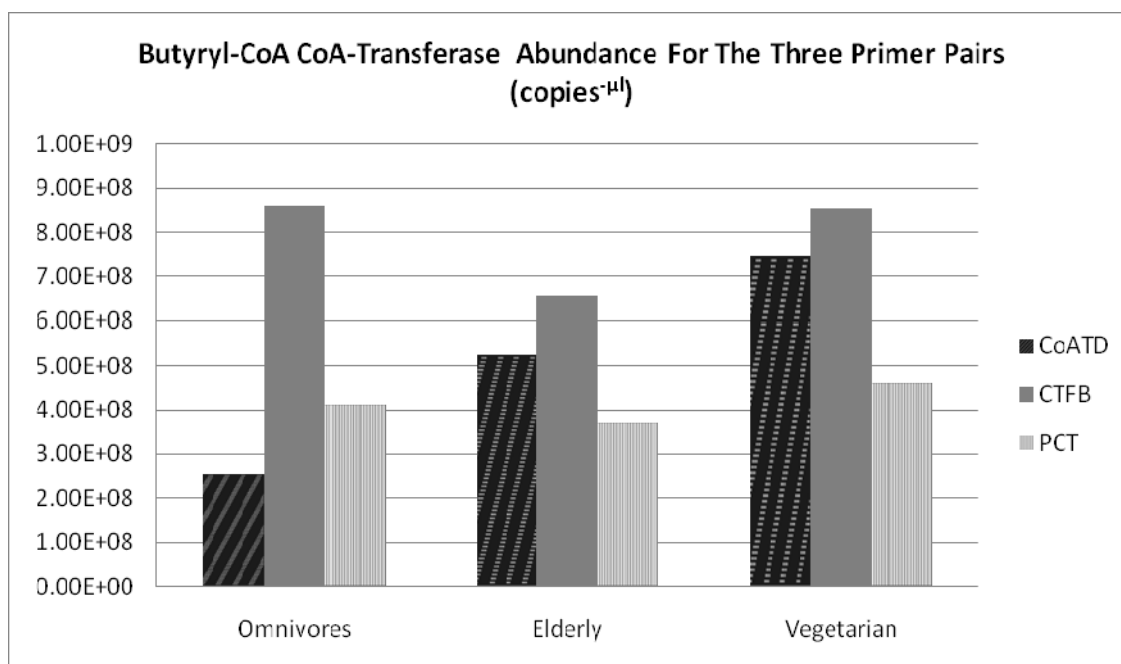


Figure 2: absolute concentration of Butyryl-CoA CoA-transferase gene abundance for the three primer pairs in group comparison

Results for SCFAs

Faecal concentrations of butyrate in specimens ranged from below detection limit up to 0.094 µmol g⁻¹ faeces (wet weight). 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, due to high inter-individual differences their values were significantly different only to Elderly.

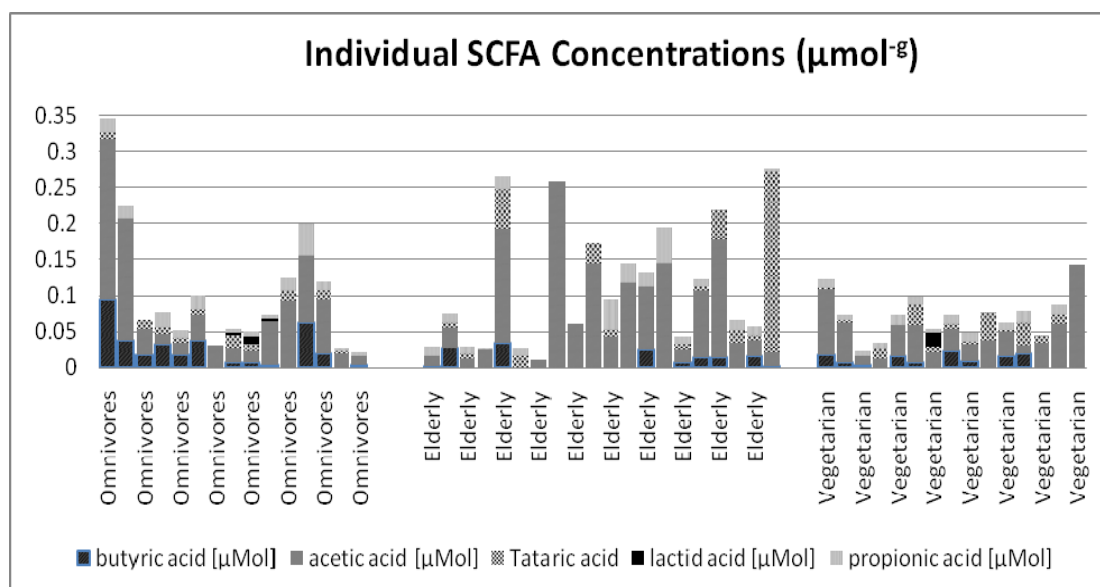


Figure 3: Individual SCFA concentrations

Relation of specimen specific Archaea

Analysis of 16S rRNA genes showed that the mean abundance of Archaea in Omnivores was highest, being more than 9 times higher than for Vegetarians. Mean values of Elderly were 2.8 times those of Vegetarians. These differences were not significant due to high inter-individual differences.

DISCUSSION

Production and consumption of SCFAs is related by microbiota diversity which is influenced by diet, age, activity and other life style factors as well as host genetics. Our group has previously reported (bla) that vegetarians have 12% more bacterial DNA in their faeces than omnivores. *Clostridium* cluster IV and *bifidobacteria* showed no significant differences for vegetarians compared to omnivores. *Bacteroides* abundance was higher for vegetarians. To study the effects of changes in microbiota and their 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 because of their potential involvement in microbial consumption of SCFAs.

Methodological Aspects

Real time PCR for CoA-transferase genes

Degenerate primers were designed by [CHARRIER et al.] for a CoA-transferase gene found in *Roseburia* sp. A2-183. Charter et al. (2004) 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 real-time PCR by comparing the melt curve peaks to a negative control (*Bacteroides thetaiotaomicron* DSM 2079) and a positive control standard (specimen PCR product, including all identical target butyryl-CoA CoA transferase gene regions). Melt curve analysis and gel electrophoresis confirmed only a single PCR product. However, the three primer pairs used do not cover all butyrate producing bacteria, as described in Flint et al. (2006), because not all bacterial Butyryl CoA transferase gene loci are known.

HPLC of SCFAs

The RI detector and the organic acid chromatographic column have regularly been used to measure SCFAs. Detection limits are influenced by limited specimen volumes, their dilution and preparation.

Weaver et al. (1997) 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 (Weaver, Tangel et al. 1997; Hallert, Bjorck et al. 2003). 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 (McFall-Ngai et al. 2007). As a consequence, faecal concentrations of SCFAs are not necessarily representative of those in the more proximal colon, and can also be affected by intestinal transit time (Lewis and Heaton 1997).

Comparative real-time PCR of Archaea

As different strains might have slightly different amplification efficiencies we express abundance of Archaea in % of control. Thus a real-time PCR product from one of the samples was used as a standard.

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

The major differences observed between the measured groups (Omnivores, Elderly and Vegetarian) were the high butyric acid levels in Omnivores compared to Vegetarian individuals. The CoATD primer set amplification of butyric acid genes was highest in Vegetarians. This unexpected relationship might be attributable to a higher abundance of bacteria in vegetarians (Liszt et al. 2009), assuming that the bacterial surplus in those individuals carries genes that are amplified by this primer pair. 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 (Liszt et al. 2009). It might well be that a vegetarian diet favours the abundance of butyrate-consuming microorganisms in the microbiota of the gut. Elderly had low butyric acid levels and low butyryl-CoA CoA transferase gene abundance, but showed the highest levels of acetic acid among all subjects. Zwielehner et al. (2009) 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 abundance, despite of high functional redundancy in the human GI microbiome (Lozupone et al. 2008).

For Vegetarians the sequences amplified with CoATD primers were significantly more abundant than in all other individuals. Also the lower levels of butyric acid found in Vegetarians does not correspond to the Butyryl-CoA CoA transferase gene abundances. The targeted genes might not be equally expressed in all individuals and the butyric acid could be removed from the gut lumen by the gut mucosa and/or microorganisms. According to Stams et al. (2005) sulfate-reducing bacteria easily outcompete methanogens for hydrogen and also grow much faster on propionate and butyrate than syntrophic consortia. Furthermore

not all bacteria might use the targeted gene Butyryl-CoA CoA transferase in the final steps of butyric acid production and the three primer pairs might not cover all butyrate producing bacteria (Flint et al. 2006).

Up to 85% of humans carry Archaea in high abundances (Buck S 2006). The incidence of Archaea was highest in Omnivores. Omnivores also had the highest levels of Butyryl-CoA CoA transferase genes. Vegetarians had the lowest Archaea abundances and highest Butyryl-CoA CoA transferase gene abundances. Elderly were in between Omnivores and Vegetarians. In contrast to findings by Abell et al (2006) we observed a trend for high abundances of Archaea in samples with high butyric acid concentrations, however no linear correlations could be found, possibly due to enormous inter-individual variability.

Our experiments showed 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 vegetarians. Elderly Austrians are reported to consume 78 g meat day⁻¹, whereas adult omnivores are reported to consume 103 g day⁻¹, 60 g more than recommended. The overall protein uptake in vegetarians matches the D-A-CH recommendations (0.8g kg⁻¹ body weight). The major difference in diets might thus be the differential uptake of nitrogen from dietary protein (Austrian Nutrition Report 2009). Approximately 10% of dietary protein reaches the colon (Schmidt R. et al. 2007). According to the 2009 Austrian Nutrition Report fibre consumption is staggered as vegetarians > omnivores > elderly. All Elderly in our geriatric subject group received prebiotic supplementation.

By combining molecular analyses with HPLC of SCFAs as well as a food and activity questionnaire, we demonstrate the profound effects of diet and ageing on the human gut microbiome. These results demonstrate that faecal butyric acid levels do not correlate with the abundance of the Butyryl-CoA CoA-transferase gene. The complexity of the colonic environment renders it difficult to use single metabolites such as butyric acid as indicators for colonic health.

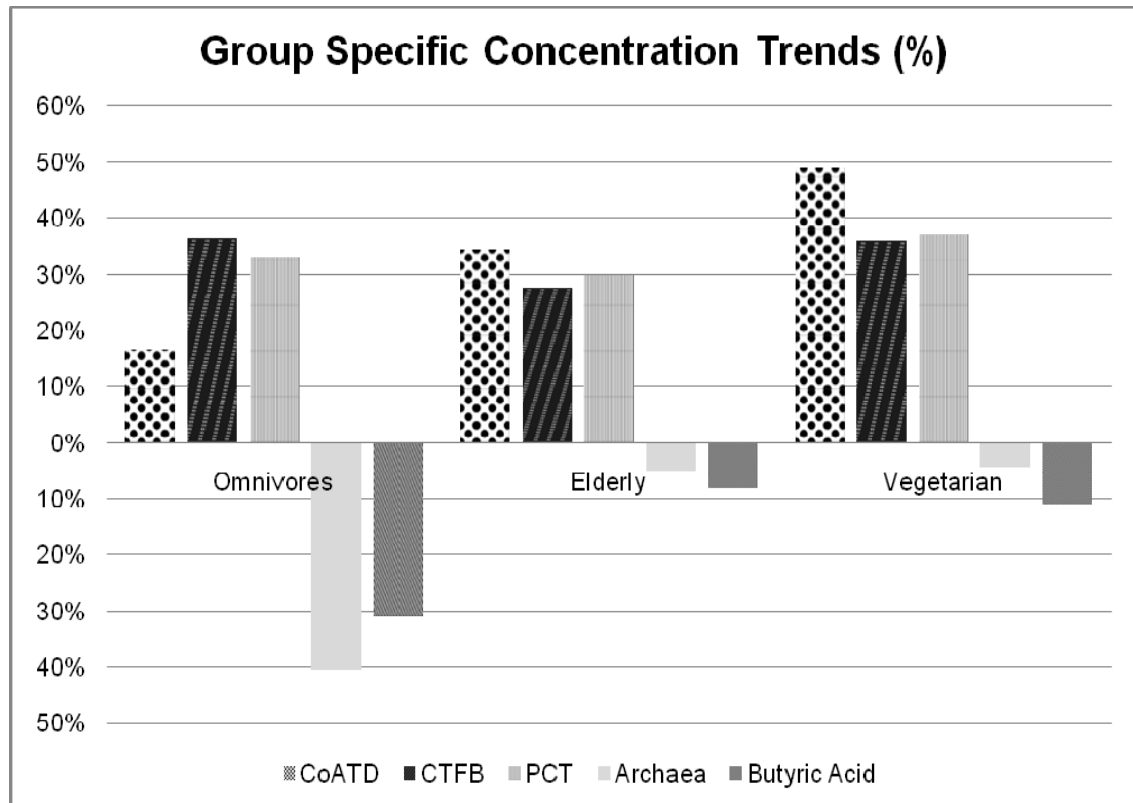


Figure 4: Group Specific Concentration Trends in 100% total of each measurement

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11.3 PUBLISHED RESEARCH ARTICLE – „COMBINED PCR-DGGE FINGERPRINTING AND QUANTITATIVE-PCR INDICATES SHIFTS IN FECAL POPULATION SIZES AND DIVERSITY OF *BACTEROIDES*, *BIFIDOBACTERIA* AND *CLOSTRIDIUM* CLUSTER IV IN INDUSTRIALIZED ELDERLY”

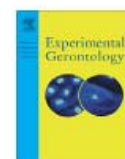
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Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of *Bacteroides*, bifidobacteria and *Clostridium* cluster IV in institutionalized elderly

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ABSTRACT

Aims: This study aimed at determining ageing-related shifts in diversity and composition of key members of the fecal microbiota by comparing institutionalized elderly ($n = 17$, 78–94 years) and young volunteers ($n = 17$, 18–31 years).

Methods and results: A combination of molecular methods was used to characterize the diversity and relative abundance of total gastro-intestinal flora, along with relevant subsets within the genera *Bacteroides*, bifidobacteria and *Clostridium* cluster IV. The institutionalized elderly harbored significantly higher numbers of *Bacteroides* cells than control ($28.5 \pm 8.6\%$; $21.4 \pm 7.7\%$; $p = 0.016$) but contained less bifidobacteria (1.3 ± 0.9 , $2.7 \pm 3.2\%$, $p = 0.026$) and *Clostridium* cluster IV ($26.9 \pm 11.7\%$, $36.36 \pm 11.26\%$, $p = 0.036$). 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.

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1. Introduction

It is estimated that between 60% and 80% of the microbial diversity resident within the human gastro-intestinal tract (GI) has yet to be cultivated (Suau et al., 1999). However, the use of culture-independent molecular methods has enabled researchers to identify important characteristics of this community. We now know that the composition of GI microbiota is highly distinct between individuals (Dethlefsen et al., 2006). Despite this variability, some general features are apparent. There appear to be 'core species' present in a majority of humans which are quite resilient to external influences (Dore, 2007). In addition to this group, there are also 'passengers' or transients, sometimes in great numbers, sometimes below detection limit (Favier et al., 2003). However, the majority of

sequences seem to be unique to some individual and not one single phylotype may be present in all humans (Turnbaugh et al., 2009). A number of factors have been identified which influence community composition. The type of bacteria introduced to the environment is determined by those associated with food sources (Duncan et al., 2007; Pryde et al., 2002). The nature and composition of non-digestible carbohydrates in a diet can further stimulate different types of bacteria (Kolida and Gibson, 2007), whilst hereditary dispositions and personal gut environmental factors may account for unique personal characteristics (Eckburg and Relman, 2007).

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

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

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

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

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

With ageing, a decrease in beneficial organisms such as *Lactobacilli* and bifidobacteria, amongst other anaerobes, and an increase in the number of facultative anaerobes (Guigoz et al., 2008) have been reported. Population cross-sectional studies with relevant numbers of healthy elderly also show age-related changes in GI microbiota. These include a consistent global increase in nonpathogenic Gram-negative bacteria (mainly *Enterobacteria*), as well as country-specific changes in bifidobacteria (Guigoz et al., 2008). This, along with a general reduction in species diversity within most bacterial groups, changes to diet and altered digestive physiologies such as intestinal transit time, may result in increased putrefaction in the colon and a greater susceptibility to disease. The aged gut is characterized by increased proteolytic activity, decreased amylolytic activity and reduced levels of SCFA (Woodmansey, 2007). Ageing is associated with reduced levels of prostaglandins such as PGE₂ and PGF₂α as shown in specimens of stomach and duodenum biopsies (Tiihonen et al., 2008). Subclinical intestinal inflammation in elderly populations has been detected and is believed to contribute to impaired immune functions, the underlying cause of mortality beyond 75–80 years of age (Guigoz et al., 2008).

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

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

2. Material and methods

2.1. Proband

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

on demand. Seventeen young healthy volunteers were aged 24 ± 2.5 years, BMI 22.68 ± 3.41 and their dietary habits were typical for Central Europe.

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

2.2. Sampling and DNA extraction from stools and type strains

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

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

2.3. Polymerase chain reaction (PCR)

PCR was used to amplify 16S ribosomal RNA gene sequences from type strains and *Bacteria* in stool samples for use in DGGE analysis and manufacture of clone libraries (see below). All reactions were carried out using a ready-to-use mastermix (Promega) with 1.5 mM MgCl₂. Bovine serum albumin (10 mg/ml, Fermentas) was added to a final concentration of 400 µg/ml, primer concentration in the reaction volume was 0.5 µM. Amplifications were carried out in a Robocycler (Stratagene).

2.4. Clone libraries

Clone libraries were constructed from stool samples to identify dominant members of the *Clostridium* cluster IV and the *Bacteroidetes*. Selected clones were then used, along with the cultured type strains, to generate appropriate reference markers for DGGE analysis. Amplifications were carried out using primer pair 27f Edwards et al., 1989) and sg-Clep-R (Matsuki et al., 2004), which are specific for members of the *Clostridium* cluster IV, and 32f and 708r (Bernhard and Field, 2000) which are specific for members of the *Bacteroidetes*. 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

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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	Pwd primer	GCG TGC TTA ACA CAT GCA AGT C	125	300	Penders et al. (2005)
	Rev primer	CAC CCG TTT CCA GGA GCT ATT		300	
	Probe	(FAM)- TCA CGC ATT ACT CAC CCG TTC GCC -(BHQ-1)		150	
<i>Bacteroides</i>	AllBac296f	GAG AGG AAG GTC CCC CAC	106	300	Layton et al. (2006)
	AllBac412r	CGC TAC TTG GCT GGT TCA G		300	
	AllBac375Bhq	(FAM)-CCA TTG ACC AAT ATT CCT CAC TGC TGC CT-(BHQ-1)		100	
Bacteria (general)	BAC-338-F	ACT CCT ACG GGA GGC AG	468	1000	Yu et al. (2005)
	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	
<i>Clostridium</i> cluster IV	sg-Clep-F	GCA CAA GCA GTG GAG T	239	400	Matsuki et al. (2004)
	sg-Clep-R	CTT CCT CCG TTT TGT CAA		400	
	Clep-P ^a	(FAM)-AGG GTT GCG CTC GTT-(BHQ-1)		200	

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

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

2.5. DGGE

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

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

cific DGGE analysis consisted of *E. faecium* DSM 20477^T, *E. coli* IMBH 252/07, *B. longum* DSM 20219^T, *B. longum* DSM 20211, *B. thermophilus* DSM 20210^T and *B. pseudolongum* DSM 20099^T. The reference marker for *Bacteroides* specific DGGE analysis was composed of *B. thetaiotaomicron* DSM 2079^T, fecal clones Bt 17 and Bt11 and *E. faecium* DSM 20477^T. The reference marker for *Clostridium* cluster IV DGGE analysis was constructed from 7 clones all representing previously uncultured species from cluster IV: Similarities with type strains from this cluster were 81.8%, 96.6%, 96.7% and 97.8% similarity with *F. prausnitzii*^T, 92.1% and 92.9% similarity with *Eubacterium desmolans*^T and 95.9% similarity with *Subdoligranulum variabile*^T.

2.6. TaqMan q-PCR

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

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

Table 2

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

Target organism	Primer pairs	Sequence (5'–3')	Ann. temp (°C)	Reference
Bacteria	27f	GTG CTG CAG AGA GTT GAT C TGG CTC AG	57	Edwards et al. (1989)
	985r	GTA AGT TCT TCG CGT T	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)
<i>Bacteroides</i>	32f	AAC GCT AGT CAC AGG CTT	56	Bernhard and Field (2000)
	708r	CAATCGGAGTCTTCGTG	56	Bernhard and Field (2000)
Bifidobacteria	g-BifdF	CTCTGGAACGGGTGG	58	Matsuki et al. (2002)
	g-BifdR	GGTGTCTTCCCGATATCTACA	58	Matsuki et al. (2002)
<i>Clostridia</i> cluster IV	sg-Clep-F-GC sg-Clep-R	see Table 1	55	

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

2.7. Statistical analysis

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

3. Results

3.1. Dietary aspects

Analysis of the participant's dietary habits indicated similar consumption patterns of fruits, vegetables and milk products in

both groups. Young volunteers stated significantly less frequent (χ^2 Test; $p < 0.04$) consumption of meat than elderly probands and regular consumption of whole grain products several times a week. The institutionalized elderly of this study did not consume any whole grain products at all but received supplements with soluble fiber (Benfiber[®], Novartis).

3.2. TaqMan-quantification

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

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

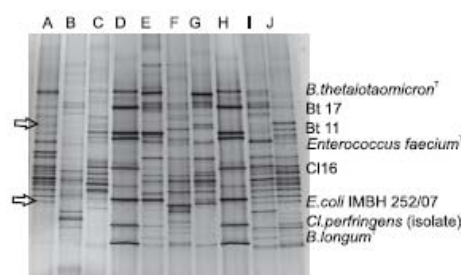


Fig. 2. PCR-DGGE bandpattern of 16S rRNA coding regions of dominant bacteria amplified with primer pair 341GC–518. Bands that were observed more frequently in young than in elderly are indicated with arrows. Organisms and sequences listed were used for the construction of the reference lanes. A, G, I,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

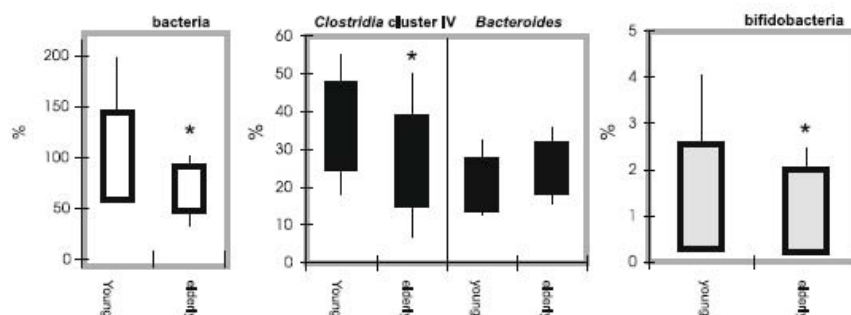


Fig. 1. Proportions of *Bacteroides*, bifidobacteria and *Clostridium* cluster IV of the total bacterial load in stool samples of institutionalized elderly and young healthy volunteers. Total amount of bacteria is depicted relative to the mean counts for healthy young young.

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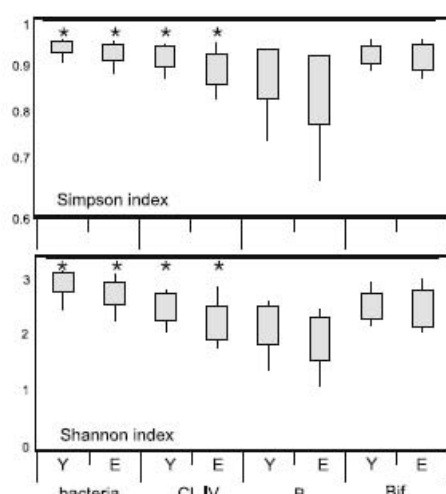


Fig. 3. Diversity indices derived from DGGE fingerprinting of 16S rRNA coding regions. Y, young; E, elderly; Cl.V, *Clostridium* cluster IV; B, *Bacteroides*; Bif, bifidobacteria; *, $p < 0.05$.

3.3. DGGE bandpattern analysis

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

3.3.1. Bifidobacteria

An average of 13 bands were observed in individual DGGE bandpatterns obtained with the primer pair specific for bifidobac-

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

3.3.2. Bacteroides

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

3.3.3. Clostridium cluster IV

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

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

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

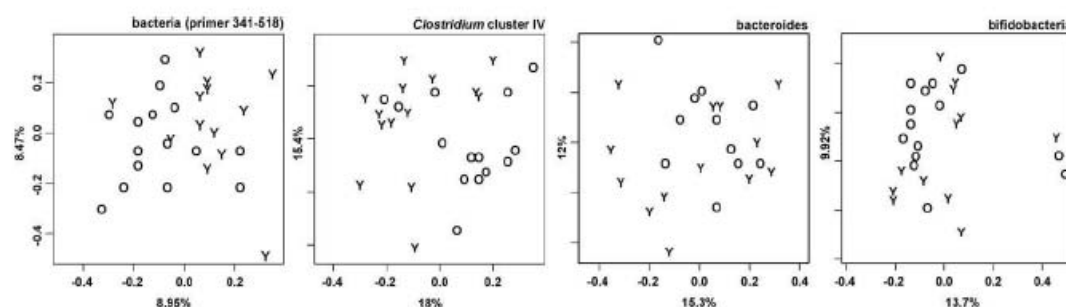


Fig. 4. PCA of DGGE fingerprints of 16S rRNA coding regions of dominant bacteria in fecal samples. Y, young; O, elderly.

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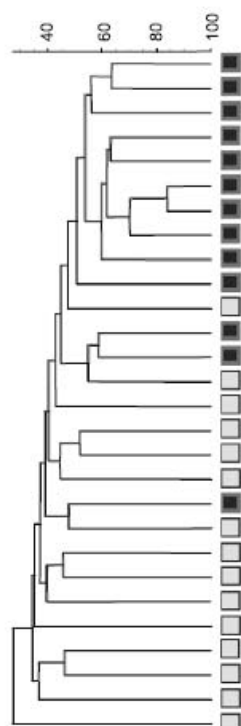


Fig. 5. UPMGA dendrogram showing clustering (Dice) based on the similarities of DGGE fingerprints of dominant bacteria obtained with primer pair 341 GC-518. $\square$ elderly, $\blacksquare$ young.

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

4. Discussion

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

Our results demonstrate some significant shifts in patterns in GI microbiota between our study groups. Faeces from the institutionalized elderly had less total Bacterial abundance and lower total Bacterial diversity than that from the young subjects. However, samples from the elderly displayed an increase in the relative abundance of *Bacteroides*, although this group tended to display less diversity than *Bacteroides* in the young. The relative abundance of the bifidobacteria

and *Clostridium* cluster IV were significantly higher in the young, and the *Clostridium* cluster IV also displayed greater diversity in the young. Furthermore, cluster analysis revealed that for all microbial groups analyzed, the members of the GI microbiota in the elderly could be considered a subset of that present in the young.

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

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

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

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

5. Conclusion

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

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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.

Acknowledgements

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11.4 POSTER PRESENTATION – 5TH INTERNATIONAL YAKULT SYMPOSIUM

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

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BACKGROUND: The composition of the gastrointestinal microbiota is highly distinctive between individuals and the majority of sequences seem to be unique to some individuals (Turnbaugh et al., 2009). Correlations between the gastrointestinal microbiota and diseases like 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. Specific genera have been associated with specific health benefits such as short chain fatty acid production by species of the genera *Clostridium*, *Eubacterium* and *Fusobacterium*. Gastrointestinal disturbances (e.g.: diarrhea) are often induced in response to cancer treatments such as chemotherapy or radiation (Abd El-Atti et al., 2009). Disturbances in the homeostasis of microbiota are discussed to favor severely pathogenic bacteria such as *Clostridium difficile*.

OBJECTIVES: The purpose of this study was to investigate changes within the GI microbiota in response to chemotherapy against different kinds of cancer. We assessed diversity and abundance of bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster IV and *XIVa* and *Clostridium difficile*.

SUBJECTS: Faeces, food frequency questionnaires and case report forms of 9 ambulant patients (ON, 60 ± 11 years) receiving chemotherapy ± antibiotic treatment were collected and analyzed at four sampling points before or after the onset of treatment in comparison to 9 gender-, age- and lifestyle-matched healthy individuals (C, 53 ± 18 years).

METHODS: We targeted 16S rRNA genes of bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster IV and *XIVa* as well as *Clostridium difficile* with TaqMan-qPCR. We assessed diversity and temporal stability of bacteria and *Clostridium* clusters IV and XIVa at the four sampling points using denaturing gradient gel electrophoresis (DGGE) fingerprinting.

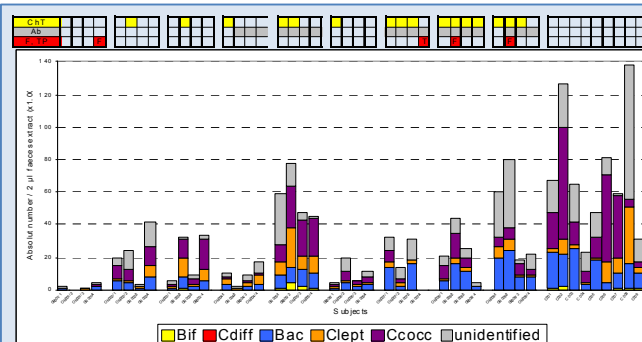


Figure 2: Absolute numbers of *Bacteroides* (Bac), bifidobacteria (Bif), *Clostridium* cluster IV (Clept) and *XIVa* (Ccocc), *Clostridium difficile* (Cdif) and unidentified bacteria analyzed by TaqMan-qPCR. ChT: Chemotherapy, Ab: Antibiotics F: fever, T: blood stem cell transplantation. Subject ON001 received chemotherapy before sampling.

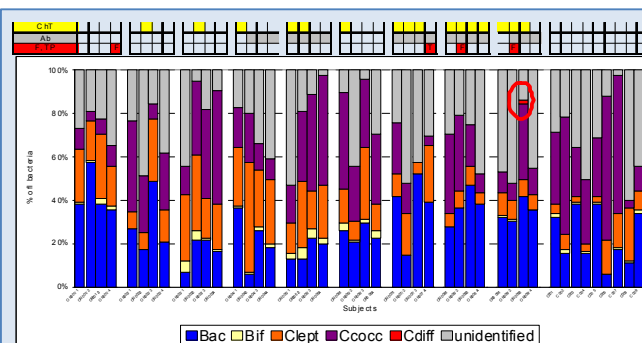


Figure 3: Percentage of *Bacteroides* (Bac), bifidobacteria (Bif), *Clostridium* cluster IV (Clept) and *XIVa* (Ccocc), *Clostridium difficile* (Cdif) and unidentified bacteria of analyzed bacteria quantified by TaqMan-qPCR. *Clostridium difficile*

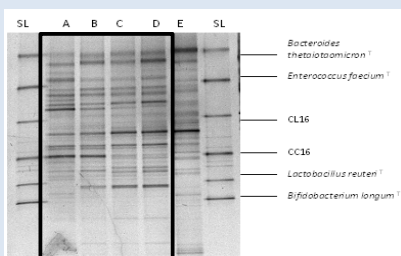


Figure 1: PCR-DGGE fingerprinting of 16S rRNA coding regions of bacteria. SL: standard lane, A, B, C and D: ON001 receiving chemotherapy, E: healthy control.

RESULTS & DISCUSSION:

- Faecal microbiota of patients receiving chemotherapy (± antibiotics) decreases significantly after cycles of chemotherapy and in comparison to healthy controls.
- After the end of chemotherapeutic cycles the magnitude of microbiota recovers within a few days sometimes even showing a "rebound-effect".
- All bacterial subgroups show high intra- and interindividual variations.
- The chemotherapeutic treatment has marginally affected the genus *Bacteroides* while the genera *Clostridium* cluster IV and *XIVa* seem to be more sensitive to chemotherapy and antibiotic treatment.
- A decreased diversity of bacteria and *Clostridium* cluster XIVa was observed in response to medical treatment compared to healthy individuals.
- The incidence of *Clostridium difficile* in subject ON009 results or is a result of a decrease of the genera bifidobacteria and *Clostridium* cluster IV.
- Monitoring of microbiota using qPCR or PCR-DGGE might be an easy and adequate tool to guide decisions for or against probiotic intervention.

11.5 ABSTRACT – 19TH INTERNATIONAL CONGRESS OF NUTRITION (ICN 2009)

CHANGES IN HUMAN FAECAL MICROBIOTA DUE TO CHEMOTHERAPY ANALYZED BY TAQMAN-PCR AND PCR-DGGE

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We analyzed the diversity and abundance of *Bacteroides spp.*, *Bifidobacteria*, *Clostridium* clusters IV and XIVa as well as *Clostridium difficile* in microbiota of individuals undergoing chemotherapeutic treatment.

Faeces of ambulant leukemic patients receiving chemotherapy $\pm$ antibiotic treatment were analyzed before and after the onset of treatment in comparison to gender-, age- and lifestyle-matched healthy controls. Diet was assessed with a food frequency questionnaire. Faecal samples were analyzed with quantitative TaqMan-PCR and qualitative PCR-DGGE fingerprinting of 16S rRNA coding regions.

A decrease of overall bacteria and *Clostridium* clusters IV and XIVa was observed in response to chemotherapy. The progression of the community changes within the gut microbiota showed high individual variations at the four time points investigated, but an overall increase of abundance of *Clostridium difficile* could be observed in leukaemia patients compared to healthy controls.

Conclusion: Overall changes of community composition within the human microbiota in response to chemotherapeutic treatment might affect the incidence of *Clostridium difficile* in leukemia patients.

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

Zwielehner, J., Liszt, K., Handschur, M., Lassl, C., Lapin, A., Haslberger, A. G.; Experimental Gerontology 2009; 44: 440-446.

Changes in human faecal microbiota due to chemotherapy analyzed by TaqMan-PCR and PCR-DGGE

Lassl, C., Zwielehner, J., Liszt, K., Hippe, B., Haslberger, A. G.
Abstract: 19th international congress of nutrition (ICN 2009)

Decreased levels of butyric acid but high amounts of bacterial Butyryl-CoA CoA-transferase in faecal microbiota, for vegetarians compared to omnivores

Hippe, B., Zwielehner, J., Liszt, K., Lassl, C., Haslberger, A. G.
Abstract: 19th international congress of nutrition (ICN 2009)