DIPLOMARBEIT

Titel der Diplomarbeit

Shifts of fecal microbiota in immunosuppressed patients following chemotherapeutic treatment analyzed by TaqMan-PCR and PCR-DGGE with special focus on 454 sequencing

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Angelika Maria Pointner

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Changes in Human Fecal Microbiota Due to Chemotherapy Analyzed by TaqMan-PCR, 454 Sequencing and PCR-DGGE Fingerprinting

Jutta Zwielehner1, Cornelia Lassl1, Berit Hippe1, Angelika Pointner1, Olivier J. Switzeny1, Marlene Remely1, Elvira Kitzweger2, Reinhard Ruckser2, Alexander G. Haslberger1*

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3. ABBREVIATIONS

A	adenine
AAD	antibiotic associated diarrhea
bp	base pairs
°C	degree Celsius
C	cytosine
CDI	*Clostridium difficile* infections
CFU	colony forming units
d	day
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
NIDDM	non insuline dependent diabetes mellitus
dNTPs	desoxyribonukleosidtriphosphate
dsDNA	double-stranded DNA
GALT	gut associated lymphoid tissue
G	guanine
GI	gastrointestinal
GIT	gastrointestinal tract
min	minute
mM	millimolar
MW	mean value
NFW	nuclease free water
PCA	principal component analysis
PCR	polymerase chain reaction
qPCR	quantitative real time PCR
RNA	ribonucleic acid
rRNA	ribosomal RNA
SCFA	short chain fatty acids
ssDNA	single-stranded DNA
STAB	standard deviation
T	thymine
V	Volt
µM	micromolar
µl	microliter

4. SUMMARY

The human intestinal microbiota represents a complex, densely populated ecosystem, which is composed of about 10^{13}-10^{14} microorganisms of more than 1000 different bacterial species. The composition of the gastrointestinal microbiota comes as a result of natural selection at both the host and microbial levels. Despite high inter-individual variation a recent study identified three robust bacterial clusters - referred to as enterotypes - that are not nation or continent specific.

Gut microorganisms carry out a variety of beneficial functions for the host such as immune modulation, fermenting unused energy substrates and producing short-chain fatty acids, regulating the development of the gut and producing vitamins. Furthermore they act as a natural barrier against harmful, pathogenic bacteria. Although the intestinal microbiota is relatively stable throughout adult life, it can be altered by host, diet, environment, bacterial factors and during various disease processes or exposure to medical treatment. These changes in composition and metabolic function of gut microbiota can influence a host's physiology and pathology and influence the development of obesity, inflammatory bowel diseases, atopic diseases, diarrhea and constipation.

The aim of this thesis was to investigate shifts in the composition of fecal microbiota of oncology patients in response to chemotherapy and antibiotics. Abundance and diversity of fecal bacteria were assessed with culture independent molecular methods.

Feces of 17 ambulant patients under immune-suppressive chemotherapy with or without concomitant antibiotics were analyzed before and after a chemotherapy cycle at four points in time in comparison to 16 gender- and agematched healthy controls. Abundance and diversity of all bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV* and *XIVa* as well as *Clostridium difficile* were assessed by targeting 16S rRNA genes with TaqMan qPCR, denaturing gradient gel electrophoresis (DGGE) fingerprinting and high-throughput sequencing.

Patients receiving chemotherapy (± antibiotics) harbored significantly less bacteria ($p < 0.05$) than healthy controls. Furthermore absolute numbers of bacteria ($p = 0.037$) were significantly lower following a single treatment of chemotherapy, but the microbiota recovered within a few days, sometimes even showing a "rebound-effect". The mean percentage of *Clostridium* cluster *XIVa* decreased significantly after administration of chemotherapy. DGGE fingerprinting showed decreased diversity of *Clostridium* cluster *IV* and *XIVa* in response to chemotherapy. *Clostridium* cluster *IV* diversity was particularly affected by antibiotics. In three out of seventeen oncology patients *Clostridium difficile* occurred following chemotherapy. Additionally, a decrease in the genera Bifidobacterium*, Lactobacillus, Veillonella* and *Faecalibacterium* was observed.

In conclusion, chemotherapy treatment alters the composition of fecal microbiota and thus may favor colonization with potential pathogens such as *C. difficile*. These changes affect and alter the functions of the GI microbiota and may contribute to severe side effects in oncology patients.

5. ZUSAMMENFASSUNG

Die Mikrobiota im menschlichen Darm stellt ein komplexes dicht besiedeltes Ökosystem dar, welches aus etwa 10^{13}-10^{14} Mikroorganismen mit mehr als 1000 verschiedenen Bakterienarten besteht. Die Zusammensetzung dieser ist ein Ergebnis natürlicher Selektion sowohl auf mikrobieller als auch auf Wirtsebene. Trotz großer Unterschiede der Mikrobiota zwischen einzelnen Individuen, konnte eine aktuelle Studie drei robuste Bakteriencluster identifizieren. Diese "Enterotypen" zeigten sich unabhängig der Herkunft der Versuchspersonen.

Die Mikroorganismen im Darm erfüllen eine Vielzahl nützlicher Aufgaben für den Wirtsorganismus, wie zum Beispiel die Fermentierung von unverdauten Nahrungsbestandteilen und die Produktion von kurzkettigen Fettsäuren. Weiters regulieren sie die Entwicklung des Darmepithels und sind an der Synthese von Vitaminen beteiligt. Die Mikrobiota dient zudem als natürliche Barriere gegen pathogene Bakterien und moduliert das Immunsystem des Wirts. Während des Erwachsenenalters ist die Zusammensetzung der Darmbakterien relativ stabil, kann jedoch durch verschiedene Faktoren beeinflusst werden. Dazu gehören Ernährung und Lifestyle sowie auch verschiedene Erkrankungen oder medizinische Behandlungen.

Veränderungen in der Zusammensetzung und somit auch der metabolischen Funktion der Mikrobiota können sich auf die Entwicklung und Pathogenese von Übergewicht, atopischen Erkrankungen, Durchfall, Verstopfung und entzündlichen Darmerkrankungen auswirken.

Die vorliegende Diplomarbeit beschäftigt sich mit der Analyse der Veränderungen der fäkalen Mikrobiota von Onkologie-Patienten während einer Chemo- und Antibiotikatherapie. Ziel war, mittels 16S rRNA basierten kulturunabhängigen Methoden den Einfluss von verschiedenen Chemotherapeutika auf die Diversität und Menge der Darmbakterien zu erfassen.

Dazu wurden Stuhlproben von 17 Chemotherapie-Patienten mit oder ohne gleichzeitiger Antibiotika-Einnahme mit 16 gesunden Kontrollpersonen ähnlichen Alters und Geschlechts verglichen. Untersucht wurden vier verschiedene Zeitpunkte vor, während und nach eines Chemotherapiezyklus. Absolute und relative Zahlen von Bakterien, *Bacteroides*, Bifidobakterien, *Clostridium* cluster *IV, XIVa* und *Clostridium difficile* im Stuhl wurden mit real time PCR (TaqMan-Detektion) erfasst. Diversitätsanalysen erfolgten mit DGGE (Denaturierungs-Gradientengel-Elektrophorese) und high-throughput Seqenzierung.

Chemotherapiepatienten (± Antibiotika) zeigten im Vergleich zur gesunden Kontrollgruppe signifikant weniger Gesamtbakterien ($p < 0,05$). Auch direkt nach einem Chemotherapiezyklus waren die absoluten Bakterienzahlen deutlich verringert ($p = 0.037$) und zudem der relative Anteil von *Clostridium* cluster *XIVa* signifikant erniedrigt. Dennoch konnte sich die Mikrobiota nach Beendigung der Chemotherapie meist wieder auf das Ausgangsniveau und bei manchen Patienten auch darüber hinaus erholen.

DGGE fingerprinting zeigte nach einer chemotherapeutischen Intervention eine verringerte Diversität von *Clostridium* Cluster *IV* und *XIVa*, cluster *IV* wurde zusätzlich durch gleichzeitige Antibiotika-Einnahme beeinflusst. Bei drei der 17 Onkologie-Patienten konnte nach der Chemotherapie ein Vorkommen von *Clostridium difficile* beobachtet werden. Gleichzeitig zeigten die Genera *Bifidobacterium, Lactobacillus, Veillonella* und *Faecalibacterium prausnitzii* einen Abfall.

Diese Ergebnisse zeigen, dass Chemotherapeutika einen erheblichen Einfluss auf die Zusammensetzung der fäkalen Mikrobiota haben können. Durch diese Veränderungen kann die Besiedlung mit potentiell pathogenen Bakterien wie *Clostridium difficile* begünstigt und die nützlichen Funktionen der Darmbakterien beeinträchtigt werden, was im Verlauf einer Chemotherapie zu schweren Nebenwirkungen beitragen kann.

6. OBJECTIVES

The human intestinal microbiota is composed of about 10^{13}-10^{14} microorganisms with an estimated mass of 1-2 kg [LEY et al., 2006] belonging to more than 1000 different bacterial species. A substantial part of these species are still to be described. The microbiota represents a complex, densely populated ecosystem, where strict principles regarding growth control and community life must exist to maintain a healthy milieu [STEINHOFF, 2005].

For health it is very important to have a balanced ratio of the different bacterial populations. Adapted to the environment gut bacteria perform a variety of tasks in the different intestinal segments from immune modulation to the supply of vitamin K1. Furthermore, they produce short-chain fatty acids which nourish the intestinal mucosa, act antiphlogistic on the one hand and protect against colon cancer on the other [HAMER et al., 2008]. Also undisputed is scientific evidence regarding the influence of metabolites on the psyche and the frame of mind [BRAVO et al., 2011].

The healthy gastrointestinal microbiota is more or less constant, but infectious diseases, severe psychological stress or poor nutrition can disturb the orderly interactions of the bacteria in the gut and allow overgrowth of potential pathogens like *Clostridium difficile* [VAN DER WAAIJ, 1989]. Chemotherapy and the use of antibiotics may also disrupt the ecological balance [NYHLEN et al., 2002]. As a matter of fact antimicrobial therapy can result in antibiotic-associated diarrhea (AAD) [KONING et al., 2008] and increases the risk of a *Clostridium difficile* infection [HENSGENS et al. 2011].

Well known side-effects of cancer treatments are immunosuppression and gastrointestinal disturbances like constipation, diarrhea and mucositis. Yet, little research has been conducted on the underlying mechanism and the shifts in microbiota.

The purpose of this thesis was to investigate changes in the composition of fecal microbiota of patients under chemotherapeutical administration with or without antibiotics at four different points in time in comparison to a healthy

control group. Sample times were before and after a single chemotherapeutic cycle, so qualitative and quantitative changes were studied over the course of time. The aim was to clarify how chemotherapeutic agents influence fecal bacteria by assessing abundance and diversity with culture independent methods. Dominant bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV*, *Clostridium* cluster *XIVa* and *Clostridium difficile* were analysed.

7. INTRODUCTION

7.1. The human gut

In the human organism the intestinal mucosa represents the largest boundary to the environment. At the same time it is estimated that the human intestine accommodates 10 times more bacterial cells than body cells [SAVAGE, 1997] and 100 times more genes than the human genome [BÄCKHED et al., 2005]. The density of microorganisms increases from the mouth to the anus. In total, 10^{14} prokaryotic organisms live in the intestine. As shown in figure 1 the bacterial numbers range from 10^{11} cell/g content in the ascending colon to 10^{7-8} in the distal ileum and 10^{2-3} in the proximal ileum und jejunum [NEISH, 2009].

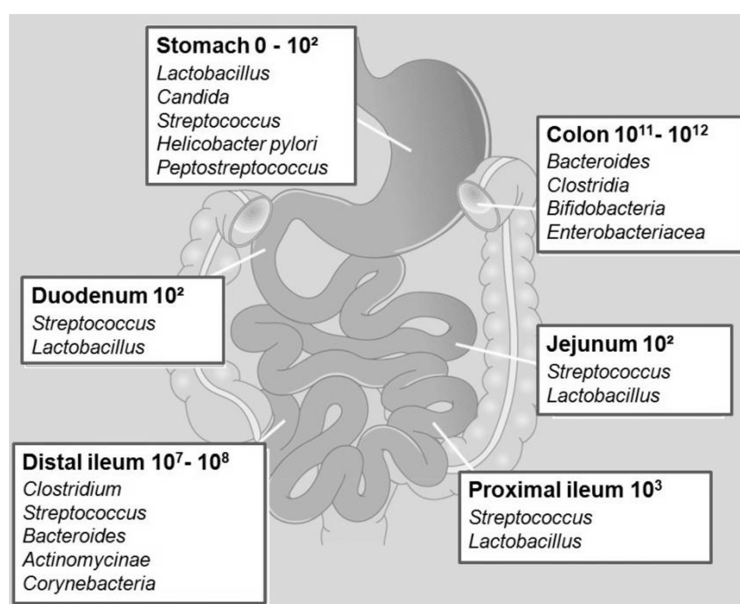

Figure 1: Population composition and numbers in cell/g content in the intestine [DEIBERT et al., 2010]

The intestinal microbiota can be pictured as a “forgotten organ”, since its metabolic activity is equal to a virtual organ [O'HARA & SHANAHAN, 2006]. The microorganisms carry out a variety of beneficial functions for the host, such as immune modulation, fermenting unused energy substrates and producing short-chain fatty acids, regulating the development of the gut, producing vitamins and furthermore, they act as a natural barrier against harmful, pathogenic bacteria. When considering the importance of microbial functions to

the host, the relationship between the human host and its intestinal microbes must in many cases be termed symbiotic rather than commensal.
The community within the human gut is extraordinarily diverse. Although bacteria predominate, it also contains archaea, yeasts and filamentous fungi [RAJILIC'-STOJANOVIC' et al., 2007]. Various recent studies [REYES et al., 2010; MINOT et al., 2011] investigated viruses as another important component. ZHANG et al. found more than 1200 viral genotypes with a density of up to 109 virions per g of dry material in human faeces [ZHANG et al., 2006]. The majority of the bacterial species in the human gut cannot be cultured, but with modern molecular methods such as sequencing of 16S ribosomal RNA from amplified nucleic acid it is possible to identify and classify bacteria. The composition of the gastrointestinal microbiota comes as a result of natural selection at both the host and microbial levels [O'HARA & SHANAHAN, 2006].
Bacteroidetes and *Firmicutes* represent with 60-90% of the total population the two dominant species [NEISH, 2009]. Frank et al. assigned with two other studies 98% of 16S rRNA sequences to only four bacterial phyla: *Firmicutes* (64%), *Bacteroidetes* (23%), *Proteobacteria* (8%) and *Actinobacteria* (3%). The remaining 2% were comprised of *Fusobacteria* and the TM7 phylum [FRANK et al., 2007].

7.2. Structural changes from infants to the elderly

The gastrointestinal tract is assumed to be sterile until birth. When the foetus passes the birth canal the bacterial colonization starts. Many factors like age of the infant, mode of delivery, dietary regime and antimicrobial treatment have an influence on the highly dynamic infant microbiota composition [PENDERS et al., 2006].

The micro-organisms that colonize the infant during birth descend from the maternal fecal, the vaginal flora and also from the surrounding environment [WALLACE et al., 2011]. The initial exposure of infants born by caesarian is mainly from the surrounding environment. However a recent study showed that the mode of delivery (vaginal-delivery or caesarian-delivery) and the feeding

habit (breast-fed or total-formula-fed) has not the big impact on the composition of gastrointestinal microbiota as always presumed. All kind of infants had similar microbial compositions by 1 month of age. This observation suggested that the colonization process may start during gestation [HONG et al., 2010].

The first colonizers of the large intestine are facultative anaerobic strains such as *Escherichia coli* and *Streptococcus spp.*. The second generation of colonizing bacteria depend mainly on the feeding profile of the infant [WALLACE et. al, 2011]. Generally anaerobe genera such as *Bifidobacterium, Bacteroides, Clostridium* and *Eubacterium* follow.

Figure 2 shows the prevalence of microbial groups at day 4 and day 120 in infants not exposed to medical interventions [EGGESBØ et al., 2011]. Almost all

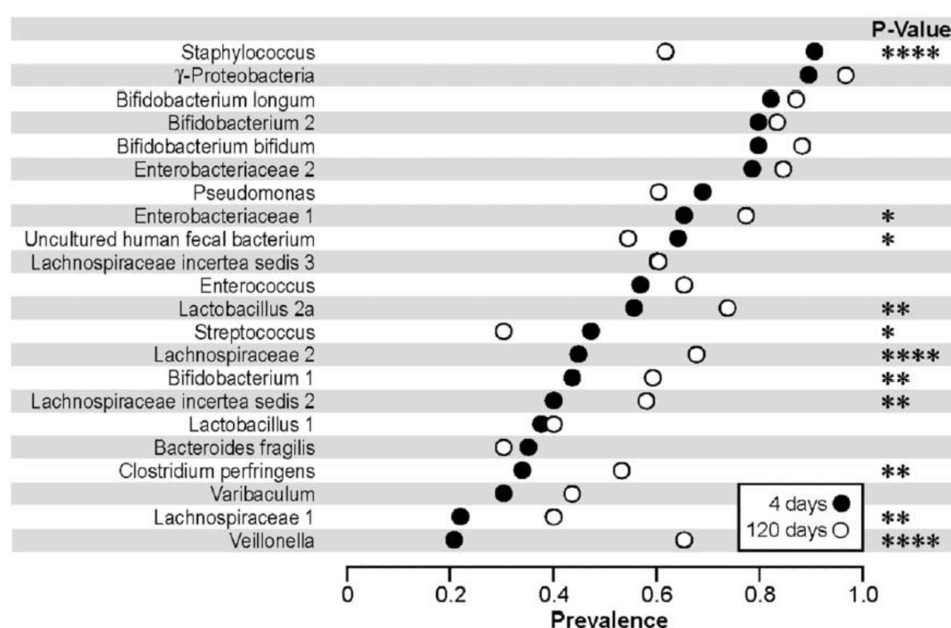

Figure 2: Prevalence of microbial groups at day 4 and day 120. Bacterial groups are ordered by mean prevalence at day 4. The p-values are calculated based on 81 infants with data for both days. **** P-value <0.0001, *** p-value <0.001, ** p-value<0.01 and * p-value<0.05 [EGGESBØ et al., 2011].

infants harbored *gamma-Proteobacteria* and *Bifidobacterium.* Associations between bacterial composition of day 4 and day 120 were observed, suggesting that early gut microbiota may influence later microbiota [EGGESBØ et al., 2011].

Breast milk is the first dietary exposure for the infant. Besides a unique combination of proteins, lipids, minerals and vitamins human milk also contains communities of bacteria and provides significant quantities of prebiotic carbohydrates other than lactose. Whereas previous works has identified lactobacilli and bifidobacteria as major phylotypes [MARTIN et. al, 2009, COLLADO et al., 2009], Hunt et al. observed *Streptococcus* and *Staphylococcus* as common genera in breast milk [HUNT et al., 2011]. The oligosaccharides in milk, which are not digestible by human infants, serve as prebiotics and are selectively fermented by the gut microbiota [GERMAN et al., 2008], providing bioactive compounds responsible for a wide range of beneficial effects for the infant gut [LARA-VILLOSLADA et al., 2007]. The final acquisition of the infant gastrointestinal microbiota takes places at weaning and a complex gut microbiota emerges [WALLACE et al., 2011].

Under normal circumstances the intestinal microbiota is relatively stable throughout adult life, but infectious diseases, medical administration, stress or poor nutrition can have adverse effects on the balanced interactions of the bacteria in the gut. In addition, age-related changes in the gastrointestinal tract, host immune system reactivity and altered nutrition interfere with the GI microbiota. Increased thresholds for smell and taste and the consequent reduced appetite, masticatory dysfunction and swallowing difficulties often result in a unbalanced, narrow diet in elderly people. With aging the amylolytic activity decreases as well as the intestinal motility, the latter implicating an increased retention time, constipation and fecal impaction [WOODMANSEY, 2006]. Furthermore, the composition of the gastrointestinal microbiota changes in elderly hosts. A general reduction in species diversity within most bacterial groups as well as a decrease in total numbers of beneficial organisms such as lactobacilli, bifidobacteria and *Bacteroides* was observed, whereas an increase

in the number of facultative anaerobes occurred [GUIGOZ et al., 2008; WOODMANSEY, 2006; ZWIELEHNER et al., 2009].

The decrease of relative abundances of important sub-dominant populations such as bifidobacteria and *Clostridium* cluster *IV* could have considerable consequences for the elderly host because of modified metabolic activities, e.g. a reduced formation of SCFAs, altered barrier function of the GI epithelium and a modified epithelial cell maturation and maintenance (ZWIELEHNER et al., 2009].

In summary, these changes in number and diversity of gastrointestinal bacteria, diet, digestive physiology such as intestinal transit time, may implicate impaired immune functions, increased putrefaction in the colon and a greater susceptibility to disease [WOODMANSEY, 2006].

7.3. Core microbiome vs. enterotypes

Microbes in the human gastrointestinal tract undergo selective pressure from the host as well as from microbial competitors, which results in a homeostasis of the ecosystem in which some species occur in high and many in low abundance [ARUMUGAM et al., 2011]. However, some low abundance species perform specialized and important functions for the host.

Various studies based on 16S ribosomal-RNA show a high microbial diversity within and between individuals. However, the knowledge about the phylum level composition in the human gastrointestinal tract is emerging. At the same time, the variation in species composition and gene pools within the human population is less clear [ARUMUGAM et al., 2011].

It was thought that there could be a 'core microbiota' of shared microorganisms in the gastrointestinal tracts of different individuals [TURNBAUGH et al., 2007], but Tap et al. showed in a later work that there is rather a identifiable 'core microbiome' of commonly shared functional gut genes than a core of commonly shared microorganisms [TAP et al., 2009].

Previous work shows that only one-third of the bacterial gene clusters that were conserved across 124 different individuals from Nordic and Mediterranean European origins could be associated with a broad functional assignment [QIN et al., 2010].

Finally, Arumugam et al., by combining 22 sequenced, fecal metagenomes of individuals from four countries, demonstrated the existence of three enterotype clusters that were not nation- or continent-specific, but vary in species and functional composition [ARUMUGAM et al., 2011]. Each of these three enterotypes are identifiable by the variation in the levels of one of three genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3). Enterotype 1 was enriched in *Bacteroides* and seemed to derive energy primarily from carbohydrates and proteins through fermentation. Enterotype 2 was enriched in *Prevotella* and the co-occuring *Desulfovibrio*, which can act in synergy to degrade mucin glycoproteins that are present in the mucosal layer of the gut. Enterotype 3 was the most frequent and is enriched in *Ruminococcus* as well as co-occurring *Akkermansia*, both known to comprise species able to degrade mucins. Enterotypes 1 and 2 were capable of biosynthesis of different vitamins. It still needs further research on environmental or even genetic factors that are causing the clustering.

7.4. Intestinal microbiota and its functions

Of the varied strategies and methods applied to study the function of the microbiota, perhaps the use of germ-free animals has offered the most revealing insights [GRENHAM et al., 2011]. Gut bacteria can be categorized as either beneficial or potentially pathogenic depending on their metabolic activities and fermentation end products. Health promoting effects of the microbiota may include the following: immunostimulation, growth inhibition of potential pathogens, improved digestion and absorption, vitamin synthesis, removal of cholesterol from the enterohepatic cycle, and decreased gas distension. Harmful effects include carcinogen production, intestinal putrefaction, toxin

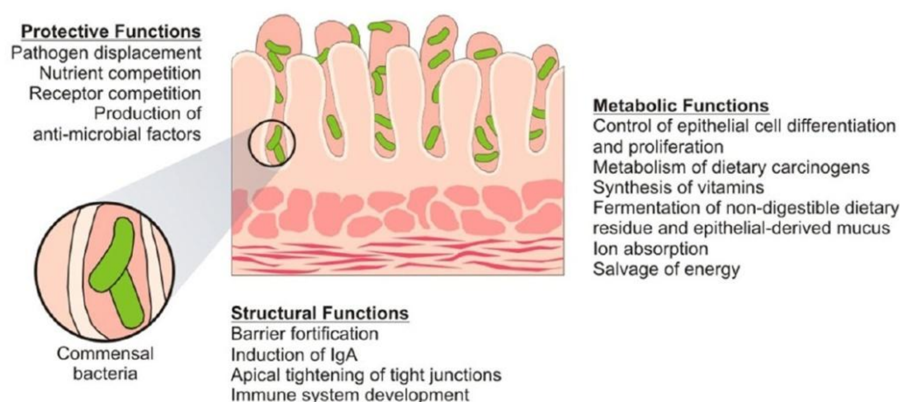

Figure 3: Functions of the intestinal microbiome [GRENHAM et al., 2011]

production, diarrhea/constipation, liver damage, and intestinal infections. [WALLACE et al., 2011].

If the gut microbiota is in good balance then it has mainly beneficial effects for the host and regulates major epithelial and immune functions of importance for gut health and health (see figure 3) [SHARMA et al., 2010; CHUNG & CASPER, 2010]. It prevents colonization of potentially pathogenic microorganisms by competing for nutrients and epithelial sites and regulates the mucosal immune system, not only educating the naive infant immune system but also serving as a relevant source of immune stimulators throughout life [ROUND & MAZMANIAN, 2009]. The GI microbiota is important for the maintenance of an intact GI barrier, which seems to be closely related to infectious, inflammatory and allergic diseases [GROSCHWITZ & HOGAN, 2009].

The microbiota also contributes to energy homeostasis and has an important role in supporting digestion and host metabolism. There are two main mechanisms by which it can maximize nutrient availability, either by fermenting indigestible food substances and so providing energy or by modulating absorption [SEKIROV et al., 2010]. A significant energy source for humans is the bacterial metabolism of dietary fiber to short-chain fatty acids such as butyrate, propionate and acetate. SCFAs in general and butyrate in particular increase growth of intestinal epithelial cells, control their proliferation and differentiation and enhance the growth of beneficial bacteria such as lactobacilli and bifidobacteria.

Furthermore, the gut microbiome is involved in synthesizing micronutrients, in breaking down dietary toxins and carcinogens and is crucial for normal GIT motility. Finally, it is responsible for producing antimicrobial compounds [SEKIROV et al., 2010], regulating fat storage and influencing angiogenesis in the intestine and epithelial turnover.

It has been shown that the gut microbiome reports to the brain via hormones and Nervus vagus and, in this way, may affect mood, behaviour and corresponding neurochemical changes in the brain [TSURUGIZAWA et al., 2009; NEUFELD et al., 2011].

The GI microbiota and the permanent challenge with bacterial antigens are crucial for the normal development and function of the gut associated lymphoid tissue (GALT) [BOUSKRA et al., 2008; ROUND & MAZMANIAN; 2009].

7.5. Bacterial communities

The microbial community within the human gut is extraordinarily diverse. Although bacteria predominate, it also contains archaea, yeasts and filemantous fungi. *Bacteroidetes* and *Firmicutes* represent with 60-90% of the total population the two dominant phyla [NEISH, 2009]. In this work the focus was on *Bacteroides,* bifidobacteria*, Clostridium* cluster *IV, XIVa and Clostridium difficile*, which are described in more detail in the following section:

7.5.1. *Bacteroides*

Bacteroides are Gram-negative, non-spore-forming, obligate anaerobic and rod-shaped bacteria and are commonly found in oral, respiratory, intestinal, and urogenital cavities of humans and animals. Some strains such as *B. fragilis* can cause potentially fatal abscesses and other infections [LI et al., 2002; PAPANILAKOU et al., 2011]. *Bacteroides spp.* are fibrolytic and involved in numerous important metabolic activities including carbohydrate fermentation and butyrate production. They possess broad glycan-degrading abilities and use a series of membrane protein complexes for catabolism of many complex

carbohydrates [MARTENS et al. 2011]. Previous studies have suggested that *Bacteroides* are characteristic for the 'ageing microbiota' [MARIAT et al., 2009; ZWIELEHNER et al., 2009]. There are contradictory results regarding the link between higher *Bacteroides* levels and obesity.

7.5.2. *Bifidobacteria*

Bifidobacteria are Gram-positive, anaerobic, branched rod-shaped bacteria. They belong to the phylum *Actinobacteria* and are commonly found in the gastrointestinal tract, vagina and mouth of mammals. Bifidobacteria ferment sugars in the intestine to produce acetic and lactic acid. Some bifidobacteria exhibit a variety of immune modulatory effects, such as being anti-inflammatory, increasing IgA secretion, and moderating allergy [HIRAMATSU et al., 2007; OUWEHAND, 2007] and have been suggested to inhibit colon cancer [SINGH et al., 1997]. Furthermore, D'Aimmo et al. suggested that certain bifidobacteria may contribute to folate uptake [D'AIMMO et al., 2012].

7.5.3. *Clostridium* cluster *IV, Clostridium* cluster *XIVa, Clostridium difficile*

Clostridia are group of Gram-positive spore-forming anaerobe to microaerophilic species and belong to the phylum *Firmicutes*. They are phylogenetically and metabolically highly diverse bacteria [MAUKONEN et al., 2006] divided into 19 clusters [COLLINS et al., 1994]. The predominant intestinal *Clostridia* belong mainly to clusters *XIVa* [*Clostridium coccoides–Eubacterium rectale* (Erec) group] and *IV* (*Clostridium leptum* group) [ECKBURG et al., 2005]. They contain saccharolytic and/or proteolytic species and are involved in metabolizing various substrates, resulting in a variety of metabolites such as fatty acids and gases. Especially short chain fatty acids formed by microbial fermentation such as butyrate have an important effect on colonic health [MAUKONEN et al., 2006].

Within ***Clostridium* cluster *IV***, the cluster *F. prausnitzii* is the most frequent and abundant [LAY et al., 2007], which has been shown to exhibit anti-inflammatory effects on cellular and TNBS colitis models [SOKOL et al., 2007].

Clostridium* cluster *XIVa contains butyrate-producing organisms such as *Roseburia* and also mucine-degrading strains such as some *Ruminococcus* species.

Anaerobic ***Clostridium difficile*** is the most common cause of antibiotic-associated diarrhea in hospitals and other healthcare facilities [ARVAND et al., 2012]. The bacterium can also be carried asymptomatically. In the past decade a dramatically increased occurrence and severity of *Clostridium difficile* infections (CDI) has been noted. Individuals who fail to mount an immune response to *C. difficile* toxin are succeptible to infection. Thus, immunosuppressed patients having a blunted ability to mount immune responses, are at a greater risk for infection [BINION, 2011]. Schalk et al. found *Clostridium difficile*-associated diarrhea as a frequent complication in patients with acute myeloid leukaemia [SCHALK et al., 2009]. Also, antibiotic exposure, that disturbs the normal intestinal flora, allows *C. difficile* to flourish and potentially result in antibiotic-associated diarrhea (AAD) [COHEN et al. 2010].

7.6. Microbiota in health and disease and modulation

The composition of the gastrointestinal microbiota can be altered by host, diet, environment, and bacterial factors and during various disease processes or exposure to medical treatment. On the other hand, changes in composition and metabolic function of gut microbiota can influence a host's physiology and pathology. Previous studies indicate that microbiota influence the development of obesity, inflammatory bowel diseases, atopic diseases, diarrhea and constipation [MAY et al., 2009; SOKOL et al.; 2008 & 2009, QIN et al., 2008]. A large body of evidence supports also a relationship between bacteria, bacterial activities and human colorectal cancer [BOLEIJ &TJALSMA, 2012].

It is now increasingly accepted that obesity is associated with substantial changes in composition and metabolic function of gut microbiota. In contrast to earlier studies the ratio of *Firmicutes: Bacteroidetes* as main link to obesity is likely to be outdated. A recent study suggested that not the ratio of *Firmicutes:*

Bacteroidetes is important, but rather the amount of SFCA produced [SCHWIERTZ et al., 2010]. Kalliomäki et al. hypothesized that early differences in fecal microbiota composition in children may predict overweight. However, further research has to be conducted in the underlying mechanisms of microbiota and weight gain [KALLIOMÄKI et al., 2008].

Therapeutic bacteria could presumably provide the same beneficial functions and activities that have evolved for the healthy microbiota. Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host. Prebiotics are defined as non-digestible dietary carbohydrates, which are able to survive stomach acid and bile, stimulate the growth and metabolism of endogenous gut bacteria and are safe for human consumption. It has been reported that probiotics and prebiotics can aid systemic and mucosal immune function, improve intestinal barrier function, modulate gut micro-ecology, and exert metabolic effects on the host and thus, ameliorate chronic intestinal inflammation, diarrhea, constipation, irritable bowel syndrome, atopic dermatitis, sepsis, food allergies, vaginitis and liver disease [WALLACE et al., 2011].

However, studies regarding the proposed effect of probiotics and prebiotics are still contradictory and further research is needed. The key to the effective use of probiotics in treating human disease is to match the correct probiotic strain with the desired clinical outcome [WALLACE et al., 2011].

7.7. Microbiota in chemotherapy and antibiotic treatment

Pathological conditions such as infections, antibiotic therapy, proton-pump inhibitory medication and immune suppression also result in shifts in microbiota, which cause severe side effects in patients. Cytotoxic chemotherapy is a common treatment for malignancies that has been in use for many years. It can cause functional and structural changes to the GIT and damages the mucosal cells [CUNNINGHAM, 1985]. Diarrhea and constipation are common harmful secondary effects, furthermore mucositis is a major problem induced by the

cytotoxic effects of cancer therapy and radiotherapy [GIBSON et al., 2006; STRINGER et al., 2007].

Previous work have been conducted on the effect of chemotherapy and antibiotics on intestinal microbiota, but there is still further research to be done on the direct effects, how these therapies interfere with the composition of the native gut microbiota. All studies found decreased numbers of total bacteria after cycles of chemotherapy. Van Vliet et al. additionally observed an increased risk of infection and overgrowth with potential pathogenic species such as *C. difficile* following chemotherapeutic and antibiotic treatment [VAN VLIET et al. 2009]. Buffie et al. showed profound alterations of intestinal microbiota following a single dose of clindamycin - a lincosamide antibiotic - and a resulting sustained susceptibility to *Clostridium difficile*-induced colitis [BUFFIE et al., 2011]. Huang et al. monitored alteration in microbiota in children with acute lymphoblastic leukemia receiving a high-dose methotrexate (HDMTX) chemotherapy. Total bacteria load and the amount of bifidobacteria, *Lactobacillus* and *E. coli* decreased significantly compared with the control group [HUANG et al., 2012].

In conclusion, chemotherapy directly affects the microbiota composition, damages the rapidly generated mucosal cells of the GI and antibiotic treatment disrupts the ecological balance, which might influence colonization resistance. Thereby the risk of infection increases and allows potential pathogens such as *Clostridium difficile* to grow [NYHLEN et al., 2002; VAN VLIET et al., 2009].

8. MATERIAL AND METHODS

8.1. Study design

Figure 4 illustrates used methods and study design of this thesis. The statistical methods of analysis are discussed in more detail in chapter 08.10.

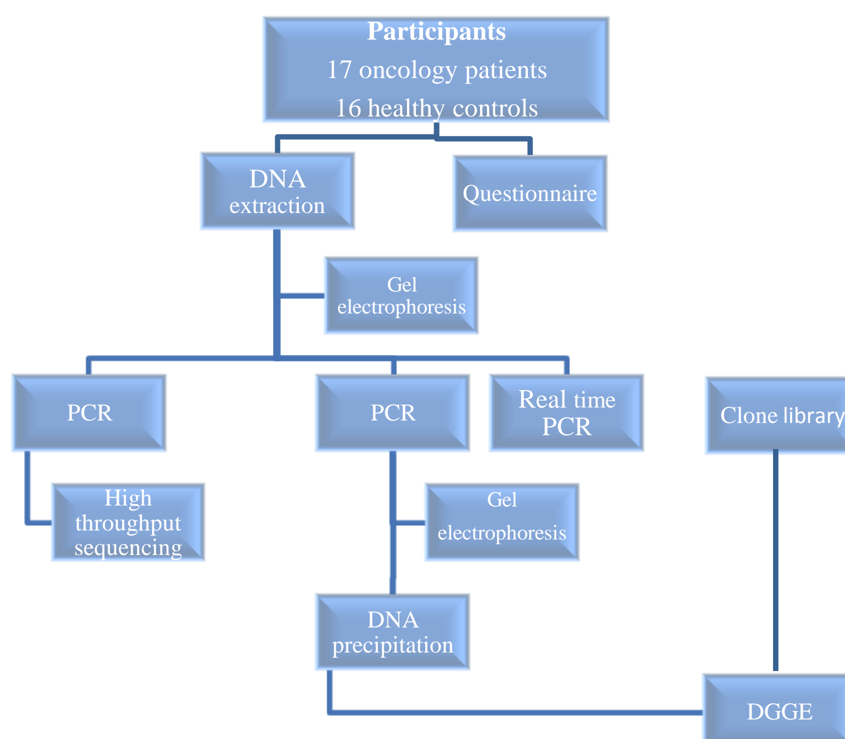

Figure 4: Study design and used methods

8.2. Study participants

This study included a study population of 33 participants. The Sozialmedizinisches Zentrum Ost (SMZO) in Vienna provided faecal samples from 17 patients under immune-suppressive, anti-malignant chemotherapy with or without antimicrobial therapy (aged 59 ± 13 y, 92 BMI 27 ± 6). The gender ratio was 47% female and 53% male. Four samples of each patient were taken within two weeks before and after a single chemotherapeutic dose. 14 out of 17 patients already had one or more chemotherapies before this treatment cycle, for three patients it was the first bout of chemotherapy. As seen in table 1 five individuals additionally suffered from diverse other diseases like hypertension,

obesity, diabetes mellitus type II (NIDDM), rheumatism and osteoporosis. Types of malignancies as well as chemotherapeutic and additional treatments like antibiotics were reported by anonymous medical records. Six patients were diagnosed with a form of leukemia, three persons suffered from a Non-Hodgkin lymphoma. Other malignancies were bowel cancers, bladder cancer, mamma carcinoma, multiple myeloma and ovarian arrhenoblastoma. The

Name	Diagnosis	Chemotherapeutic treatment	Antimicrobial treatment	Additional diseases
P01	urothel carcinoma	gemcitabine, cisplatinum		
P02	plasmocytoma, multiple myeloma	bortezomib, dexamethasone		rheumatism
P03	Non-Hodgkin lymphoma	bendamustine		diabetes II, adipositas, hypertension
P04	ovarian fibroma	taxol, carboplatin	levofloxacin	
P05	multiple myeloma	bortezomib, doxorubicin, dexamethasone	cotrimoxazole	osteoporosis
P06	mamma carcinoma	pegylated liposomal doxorubicin hydrochloride, gemcitabine		
P07	Non-Hodgkin lymphoma	high dose radiation therapy and peripheral blood stem cell transplant, cytarabine, etoposid, carmustine	cotrimoxazole, piperacillin, tazobactam	
P08	monozytic leukemia	cytarabine, idarubicin	cotrimoxazole, piperacillin, tazobactam	
P09	acute leukemia	high dose Ara-C, radiated erythrocyte concentrate, cytarabine	piperacillin , tazobactam	
P10	Non-Hodgkin lymphoma	ifosamid, etoposid, methotrexat	levofloxacin	
P11	Acute lymophoblastic leukemia	cytarabine, methotrexat		adipositas, hypertension
P12	small intestinal tumor	cetuximab		
P13	rectal tumor	capecitabine, oxaliplatin		
P14	thymus tumor	taxol, carboplatin, bevacizumab, radiation		
P15	Acute lymophoblastic leukemia	cyclophosphamide, methotrexate, doxorubicin, cytarabine, vincristine		NIDDM
P16	Acute lymophoblastic leukemia	cytarabine, mitoxantrone		
P17	colon tumor	oxaliplatin, capecitabine, bevacizumab, irinotecan, monoclonal antibodies		

Table 1: Oncology patients, malignancies, additional diseases and treatments

chemotherapeutical administration included bendamustin, bortezomib, cetuximab, cytarabin, dexamethosane, doxorubicin, etoposid, gemcitabine, irinotecan and melphalan. Six Patients (P04, P05, P07, P08, P09, P10) furthermore received antibiotics (Table 1).

The control group consisted of 16 healthy individuals (aged 65 ± 18 y, BMI 24 ± 5), 56% female, 44% male. To consider the natural fluctuations of a healthy microbiota in the research, a part of the control group had to provide several samples. Stool samples of C01-C07 were collected at three or four points in time within two weeks, while samples of C08-C16 were collected just once. Exclusion criteria for healthy controls were antimicrobial treatment, chemotherapeutic administration and the intake of pre- and probiotics at least three months before sample collection.

All subjects had to answer a questionnaire assessing their dietary habits, body constitution (age, gender, body length, weight), health status (chronic and acute diseases) and life-style aspects (e.g. alcohol consumption and physical activity). All study participants gave written informed consent. Approval was obtained from the Viennese Human Ethics committee.

8.3. Sampling and DNA extraction

Stool samples were immediately brought to the laboratory and stored at –70°C after sampling. 180-220 mg of each frozen sample were measured out and homogenized with ASL buffer in a bead beater (Mini-Beadbeater-8) twice for 45s with one intervening minute on ice. Further extraction steps were performed using the QIAamp® DNA Stool Mini Kit (QIA-GEN) following the manufacturer's protocol (see chapter 11.1.). Quality of DNA extraction was verified by gel elektrophoresis in 1% agarose gel. Thereafter DNA was stored at -20°C until the analysis was conducted.

8.4. Type strains

Cloned sequences and type strains, which are known to be a part of the human gastrointestinal microbiota were set as standards in the real-time PCRs and were used to create a standard lane marker for DGGE analysis. For latter following type strains were used: *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.

8.5. Clone library

The process of cloning in molecular biology refers to the creation of copies of a DNA fragment of interest. Figure 5 shows a schematic overview of the process.

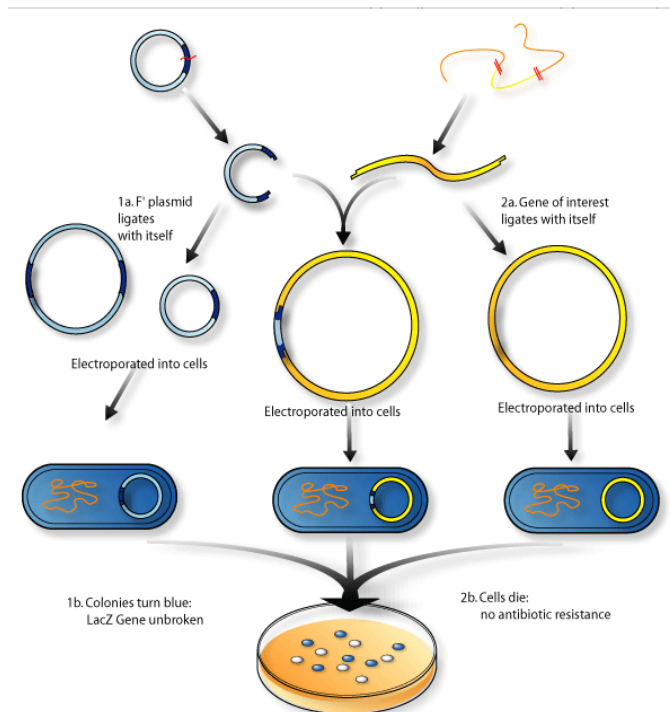

Figure 5: Procedure of molecular cloning [BIOMINEWIKI, 2011]

The amplified DNA fragment is inserted into a vector (p-GEM easy, Promega, Austria) containing an ampicillin resistance gene. Next, the vector with insert is inserted into competent *E.coli*, this is termed transformation. If a bacterial cell has incorporated the plasmid, it will grow on a LB plate containing ampicillin. With the help of blue-white screening bacteria with inserts can be detected. These bacteria can now be grown in large amounts and the target DNA is isolated again by PCR.

The object of the clone library in this study was to design a standard lane marker for the DGGE analysis and to identify dominant members of the *Clostridium* Cluster *XIVa*. For these purposes a PCR was performed with primers 195-F [MEIER et al., 1999] and Ccocc-R [MATSUKI et al., 2004] and the amplified products were inserted into a p-GEM Easy Vector (Promega)

following the instructions of the manufacturer (see also chapter 11.2.). Four LB/ampicillin/IPTG/X-Gal plates were prepared for each ligation reaction and one plate for determining transformation efficiency. 2x100 µl of each diluted transformation culture were plated and incubated at 37° over night. On the third day of the cloning procedure plates were screened for blue and white colonies. White colonies contained vectors and inserts, whereas blue colonies had no inserts. Then fifty clones were picked, transferred to clean Eppendorf tubes and amplified with Promega primers T7 and Sp6. The fragment length was checked on a 2% agarose gel. Finally, the clone inserts were sequenced (DNA confidence, Vienna) and corrected for primer and vector sequences (CodonCodeAligner). The taxonomical identification was performed with the online tools of the ribosomal database project (http://rdp.cme.msu.edu/). The clone library with the purpose of creating a standard lane marker for DGGE analysis of *Clostridium* cluster *IV* has previously been described [ZWIELEHNER et al., 2009].

8.6. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a molecular technique with the purpose of amplifying a specific region of a DNA strand (the DNA target). PCR makes it possible to produce thousands to millions of copies out of a single piece or a few copies of the target DNA. In contrast to cloning no bacteria are needed to amplify DNA in PCR. The PCR reaction requires following components:

- A DNA template containing the target sequence

- DNA polymerase: A heat resistant enzyme that can generate new strands of DNA complementary to the target region. The most commonly used DNA polymerase is *Taq* DNA polymerase, which was originates from the thermophilic bacterium *Thermus aquaticus*. Since DNA polymerases can only add new nucleotides to an existing strand of DNA, two primers are needed as starting points.

- Primers are short single-stranded oligonucleotides which are the reverse complement of a region of template or target DNA. Before using PCR it is necessary to know the exact sequences that lie on either side of both ends of the target region, to design two fitting primers for the PCR reaction.

- Nucleotids (dNTPs) are single units of the bases adenine, thymine, guanine, and cytosine. They are essential to synthesize new DNA strands.

- Buffer and $MgCl_2$ are needed as polymerase cofactors as well as bovine serum albumin (BSA) in some cases. Distilled water is used to fill up to the required reaction volume.

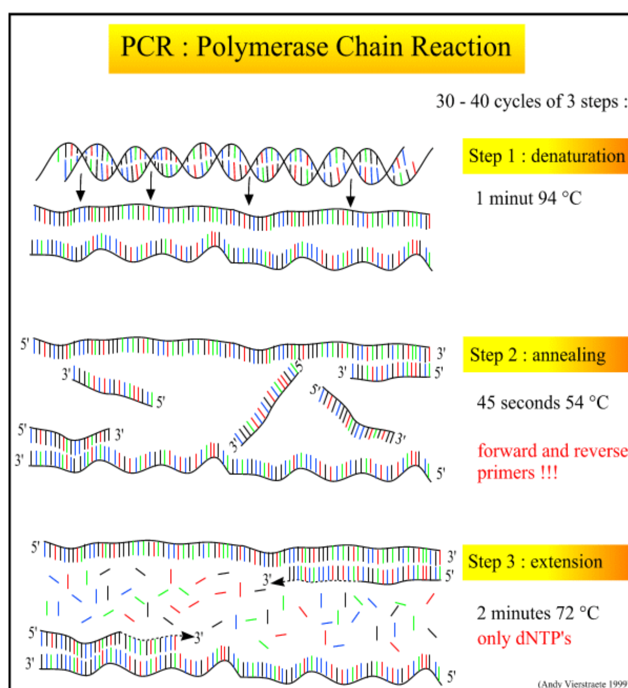

Figure 6: Polymerase chain reaction [VIERSTRAETE, 1999]

A PCR reaction is actually composed of more than 40 cycles, with each cycle consisting of three recurring temperature steps (figure 6). During initial denaturation, the reaction mix is heated to a temperature of approximately 94-96° C and held for several minutes to denature the original DNA strands and activate the heat-stable polymerase. Then 20-40 cycles follow. During denaturation the reaction mixture is heated to approximately 95°C for 20-30 seconds, which results in melting of the DNA template yielding single-stranded DNA molecules. The denaturation is followed by the annealing step, where the temperature is lowered to let the primers anneal to the complementary regions on the single stranded DNA template. The polymerase binds to the primers and synthesizes new DNA strands using dNTPs in elongation step at a temperature optimal for the polymerase. After the last cycle a final elongation step may be used to assure

that any remaining single-stranded DNA is fully elongated. PCR reactions are exponential, therefore the amount of DNA target should be doubled in each PCR cycle under optimum conditions.

Agarose gel electrophoresis is performed to check whether the amplified DNA fragment has the right fragment length. The determination of the size of PCR products is made with a special DNA ladder, which is comprised of DNA fragments of known size and is applied on the gel next to the PCR products.

PCR experiments in this study were carried out in a Robocycler (Stratagene) amplifying 16S rRNA gene sequences from bacteria in stool samples, type strains and cloned sequences for DGGE analysis and to create a clone library using group and kingdom-specific primers (see Table 2). The 16S rRNA is a component of the 30S subunit of ribosomes in prokaryotes. The 16S ribosomal RNA gene is highly conserved between different species of archaea and bacteria, that is why it is commonly used for phylogenetic studies among prokaryotes.

The PCR reaction mixture consisted of following components:

- ready-to-use mastermix (Promega) containing 1,5mM $MgCl2$
- 500nmol/L of both primers
- 2µl template
- For faecal samples, bovine serum albumin (Fermentas) was added with a final concentration of 400µg/ml

8.7. PCR-DGGE fingerprinting

Denaturating gradient gel electrophoresis is a form of electrophoresis that uses a chemical gradient to denature 16S rRNA gene fragments as they move across an polyacrylamide gel. The chemical denaturants used are formamide and urea. During DGGE PCR products are separated sequence-specifically on the basis of their melting behavior, which depends on the GC content of the fragments and length. PCR products of same length are separated according to their base sequence, by this means a separation with a difference of a single

nucleotide is possible. For the preceding PCR special primers are used containing a GC-clamp, which is an approximately 40 base pairs region consisting mainly of guanine and cytosine and prevents DNA fragments from melting completely. To identify the PCR products the resulting bands on the DGGE gel are compared with references lanes consisting of amplified DNA fragments of known bacteria. Some microorganism like lactobacill*i*, some fungi and *Bacteroides thetaitaomicron* produce several bands due to their multiple 16S rRNA operons [HIPPE et al., 2011]. This results in a pattern of bands on the gel. An analysis of the bands can be carried out with a special software (Gel Compare II, Applied Maths NV, Belgium). DNA fragments can also be cut out and sequenced.

PCR–DGGE was performed as previously described by Muyzer et al. [MUYZER et al., 1993]. Table 2 shows used primer pairs and annealing temperature for bacteria, *Clostridium* cluster *IV* and *Clostridium* cluster *XIVa*.

Target organism	**Primers**	**Sequence (5' - 3')**	**Annealing temperature (°C)**	**Reference**
Bacteria	341-F-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
Clostridium 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	
Clostridium 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 2: Primer pairs for PCR-DGGE fingerprinting of 16S rRNA coding regions

PCR products had to be precipitated for the DGGE due to the fact that 100 µl is too large a reaction volume for the slots of the gradient gel. For the precipitation -20°C cold ethanol was added to the PCR product using ten times more ethanol than product. Then the ethanol-PCR product mix was incubated at -20°C overnight and after that centrifuged for 20 minutes. The supernatant was discarded and the precipitate was dried at 37°C for 24 hours. Finally it was resuspended with 15µl NFW + 5µl loading dye. For preparation of the gradient

gels a gradient mixer (Hoefer SG 30) and a peristaltic pump were used. After resuspension the precipitated DNA was injected into the slots of the gel using a Hamilton syringe. Run time and gradient varied according to species (table 3). Standard lane markers as described above were used on each gel to ensure reliable gel-to-gel comparison. Gels were stained with GelRed and photographed under ultraviolet light. The bandpattern was analysed using GelCompareII (see also chapter statistical analysis).

Target	**Gradient**	**Runtime (hours)**	**Volt (V)**	**Temperature (°C)**
Bacteria	30-60%	9	130	60
Clostridium cluster *IV*	30-50%	5	200	60
Clostridium cluster *XIVa*	35-50%	7	200	60

Table 3: DGGE and gel settings of analyzed groups

8.8. Quantitative measurement – TaqMan-qPCR

The quantitative real-time polymerase chain reaction (qPCR) is used for absolute or relative quantification of microbial DNA [HIPPE et al., 2011] or to be precise to amplify and simultaneously quantify the DNA target. In contrast to endpoint PCR, qPCR allows monitoring the amplification in real time. Sequence-specific DNA probes with fluorescent reporters (eg. TaqMan Probes) or non-specific fluorescent dyes intercalating with dsDNA (SybrGreen) are used. During qPCR the fluorescence emission is directly proportional to the quantity of PCR product. The C_T-Value (cycle threshold value) indicates the cycle number at which the fluorescence signal exceeds the fluorescence threshold.

TaqMan probes are oligonucleotides consisting of approximately 20-30 bases and anneal to an internal region of the PCR product. They are labeled with a fluorophore (reporter) on the 5'end and a quencher on the 3' end. The quencher prevents emission of any fluorescence as long as quencher and reporter are in close proximity. When the DNA polymerase extends the primer and replicates

the PCR product, the bound TaqMan Probe degrades due to the polymerase's exo-polymerase activity and the fluorophore is released. This ends the activity of the quencher and the reporter starts to emit fluorescence.

In this study quantification of bacteria and bacterial subgroups was performed with qPCR using TaqMan probes. Analyses were performed in a Rotorgene 3000 (Corbett Life Science). The primers and probes for detection were based on 16S rRNA gene sequences acquired from different references (see Table 4). Optimal annealing temperatures of primer pairs were ascertained as previously described by Lassl 2009 [LASSL, 2009].

Target	Primer/probe	Sequence	Size
all Bacteria	BAC-338-F	ACT CCT ACG GGA GGC AG	468
	BAC-805-R	GAC TAC CAG GGT ATC TAA TCC	
	BAC-516-P	(6-FAM)-TGC CAG CAG CCG	
Bacteroides	AllBac296f	GAG AGG AAG GTC CCC CAC	106
	AllBac412r	CGC TAC TTG GCT GGT TCA G	
	AllBac375Bhqr	(6-FAM)-CCA TTG ACC AAT ATT CC CAC TGC TGC CT-(BHQ-1)	
Bifidobacteria	Bif-F	GCG TGC TTA ACA CAT GCA AGT C	125
	Bif-R	CAC CCG TTT CCA GGA GCT ATT	
	Bif-P	(FAM)- TCA CGC ATT ACT CAC CCG TTC GCC -(BHQ-1)	
Clostridium* cluster *IV	sg-Clept-F	GCA CAA GCA GTG GAG T	239
	sg-Clept-R	CTT CCT CCG TTT TGT CAA	
	Clept-P	(FAM)-AGG GTT GCG CTC GTT-(BHQ-1)	
Clostridium* cluster *XIVa	195-F	GCA GTG GGG AAT ATT GCA	538
	Ccocc-R	CTT TGA GTT TCA TTC TTG CGA A	
	Ccocc-P	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)	
Clostridium difficile	Cdiff-F	TTG AGC GAT TTA CTT CGG TAA AGA	151
	Cdiff-R	TGT ACT GGC TCA CCT TTG ATA TTCA	
	Cdiff-P	(6-FAM)-CCA CGC GTT ACT CAC CCG TCC G-(BHQ-1)	
*modified forward primer			

Table 4: 16S rRNA gene-targeted group-specific primers used in this study

The reaction mixture of 10µl was composed of 5µl Taq-Man SensiMix DNAKit (Quantance), 2 µl of sample DNA, 1µl of each primer and 1µl Taq-Man-probe. Primers and probes were diluted to a specific concentration with nuclease free

water (see table 5). Samples were analyzed in duplicates and the mean value was used for calculations. As seen in table 5 amplification programs differed depending on primers and probes. To determine the sensitivity of the real-time TaqMan assay and to construct standard curves, serial 10-fold dilutions of standards were subjected to PCR. Standards used included *Bacteroides thetaiotaomicron*^{T}, *Bifidobacterium longum ssp. longum*^{T} and *Clostridium difficile*^{T}, clones CL 16 (*Clostridium leptum* 16) and B 34 plasmid DNA (*Clostridium coccoides 34*). For universal bacterial primers a known faecal sample was set as standard.

Target	Standard	Primer/probe	Concentration	Hold	Number of cycles
all Bacteria	standard mix	BAC-338-F	10pmol/µl	95° 10min	40
		BAC-805-R	10pmol/µl		
		BAC-516-P	2pmol/µl		
Bacteroides	RK *Bacteroides thetaiotaomicron*	AllBac296f	3pmol/µl	95° 10min	40
	(DSM 2079)	AllBac412r	3pmol/µl		
		AllBac375Bhqr	1pmol/µl		
Bifidobacteria	RK *Bifidobact. longum sp. longum*	Bif-F	3pmol/µl	95° 10min	40
	DSM 20219	Bif-R	3pmol/µl		
		Bif-P	3pmol/µl		
Clostridium* cluster *IV	Clone 16 (*C.lept*.)	sg-Clept-F	4pmol/µl	95° 10min	40
		sg-Clept-R	4pmol/µl		
		Clept-P	4pmol/µl		
Clostridium* cluster *XIVa	B 34 plasmid DNA (*C.cocc*.)	195-F	5pmol/µl	95° 15min	40
		Ccocc-R	5pmol/µl		
		Ccocc-P	1,5pmol/µl		
Clostridium difficile	30 19 68 DNA (*C.diff*)	Cdiff-F	10pmol/µl	95° 10min	40
		Cdiff-R	10pmol/µl		
		Cdiff-P	2pmol/µl		

Table 5: Concentration of primers, PCR programs and standards

DNA concentrations of *Bacteroides thetaiotaomicron*^{T}*, Bifidobacterium longum ssp. longum*^{T} and *Clostridium difficile*^{T} were determined using a nanodrop spectrophotometer. The number of DNA copies per microliter was then calculated by use of the mean G and C content of each strain. Clones CL16 and plasmid DNA B34 were quantified by determination of colony forming units

(CFU) per µl. Absolute quantification was calculated by comparing C_t-values of test samples to standard curves. For comparison of different runs, a control sample was used in each run. Relative quantification of bacterial subgroups was determined by comparison of total rRNA gene copies amplified with universal bacterial primers BAC-338-F and BAC-805-R.

8.9. High throughput sequencing

Pyrosequencing is an enzyme-catalysed cascade with a light-reaction at its end indicating which nucleotide has currently been incorporated. The method relies on detecting the activity of DNA polymerase while synthesizing the complementary strand of the target DNA. Solutions of A, C, G and T nucleotides are successively added and removed to the immobile template DNA in the reaction. When the complementary nucleotide solution anneals to the first unpaired base, a chemiluminescent signal is emitted. The sequence of the target DNA can be determined by detecting these light signals.

454 life sciences has developed a whole genome high-throughput-sequencing technology by integrating pyrosequencing with their PicoTiterPlate (PTP) platform. Simultanous amplification of approximately 300,000 PCR templates is possible [LEAMON et al, 2003]. In the beginning of the 454 sequencing process the DNA template is nebulized into double stranded fragments sized between 300 and 800 basepairs. Then, DNA fragments are ligated to two different oligonucleotide adapters (A and B), which serve as binding sites for primers. DNA fragments are now denaturated and fixed to microscopic beads. Since there is a surplus of beads, statistically just one DNA fragment binds to one bead. After adding into a water-in-oil emulsion, little reaction volumina called microcontainer emerge, each containing one bead and one ssDNA template. The DNA within the microcontainers is then amplified using emulsion PCR. Each DNA-bound bead is placed into a well on a PicoTiterPlate. A DNA bead incubation mix (with DNA polymerase) and an enzyme mix (with sulfurylase and luciferase) is added and the PicoTiterPlate is then placed in to the GS FLX system for sequencing. Pyrosequencing starts and light signals are detected

with a CCD camera. A 10-hour run of sequencing results in about 400,000 reads of 250–450 bp in length. An average quality score of greater than 99.5% accuracy rate is reached [DROEGE AND HILL, 2008].

High throughput sequencing was performed with four samples of P09 and P11, before and after chemotherapy. Samples were amplified with primer pair 525F (5'-TCAGCAGCCGCGGTAATAC -3') and 926R (5'-TCCGTCAATTCCTTTGAGTTT -3') using a high-fidelity DNA polymerase (Phusion®, Finnzymes, Thermo Fisher Scientific). After 454 barcode sequencing (submission to AGOWA, Berlin, Germany) a total of 113000 reads resulted. Sequences were trimmed and aligned using the online tools of the pyro pipeline of the ribosomal database project (http://rdp.cme.msu.edu/). Sequences shorter than 151 bp were discarded, thus we had 3886 to 6811 sequences per sample of 366 to 368 bp. The *Peptostreptococcaceae* were analyzed in more detail. A phylogenetic tree was constructed by grouping of identical sequences. All analyses were performed with the ribosomal database project pyro pipeline (http://rdp.cme.msu.edu/).

8.10. Statistical analysis

Statistical evaluation of relative and absolute abundances of total bacteria and bacterial subgroups were performed using the OriginPro Version 8 (OriginLab, Northampton, MA). Here, differences between the two test groups (chemotherapy patients and healthy controls) and changes between all sample points within the groups were compared. The nonparametric Mann-Whitney U test and the two sample t-test were applied for comparisons between two groups of independent ordinal and interval values. By this means absolute numbers of all bacteria and absolute and relative numbers of subgroups in oncology patients and healthy individuals were compared. Related data within the groups were analysed using the paired sampled t-test and nonparametric Wilcoxon signed rank test. P values <0.05 were considered statistically significant.

QPCR results were plotted in heatmaps to show the decline of bacteria in T1, the time point immediately after chemotherapy. Heatmaps are graphical two dimensional tables were the values are represented as boxes of different colorshades. In this study values for heatmaps have been z-scored for presentation clarity. Z scores indicate how many standard deviations a value differs from the mean. Z-scores are calculated by subtracting the mean value from the single values and dividing that result by the standard deviation of all of the measured values, according to the formula [CHEADLE et al, 2003]

$$\textbf{Z score} = (\textbf{absolute number of bacteria}_{G} - \textbf{mean number}_{G1...Gn})/\textbf{SD}_{G1...Gn}$$

PCR-DGGE fingerprint analysis and applied principal components analysis (PCA) were performed using GelComparII (www.applied-maths.com). PCA extracts underlying components of samples according to their variance. Shannon diversity index was calculated as previously described by Zwielehner [ZWIELEHNER et al., 2009]. Shannon index is defined as $H = -\Sigma\ p_i \ln p_i$, where p_i is the proportional abundance of species *i*. The higher the Shannon index, the higher the diversity.

9. RESULTS

9.1. Dietary aspects and life-style

All study participants had to answer a food frequency questionnaire to assess their dietary habits. There were no significant differences in patients and healthy controls regarding their consumption of vegetables, grains and milk products. Patients undergoing chemotherapeutical treatment showed a lower intake of fruits, alcohol and whole grain products. Exercise levels in both groups were comparable.

9.2. TaqMan-quantification

Absolute numbers of bacteria and relative percentages of bacterial subgroups were analysed with TaqMan qPCR to investigate the influence of chemotherapeutical treatment with or without antibiotics on the human GI microbiota.

9.2.1. Absolute quantification of total bacteria and bacterial subgroups

Table 6 and 7 show absolute numbers of total bacteria and bacterial subgroups related to 2µl faeces extract of all study participants at the respective sampling times.

Controls	All bacteria	*Bacteroides*	Bifidobacteria	*C.* cluster *IV*	*C.* cluster *XIVa*	*C. difficile*
C01/1	437293.31	61137.23	244.85	133999.13	77472.39	0.00
C01/2	840409.88	53903.97	465.70	326262.04	231671.36	0.00
C01/3	1291348.28	124235.17	640.82	552740.21	396257.81	0.00
C02/1	856579.44	207233.10	0.24	135967.80	223387.78	0.00
C02/2	1280565.30	321264.60	1.19	171567.81	293190.19	0.00
C02/3	249524.26	63623.44	0.74	32472.41	75892.72	0.00
C03/1	751607.10	557747.57	991.00	111468.88	57415.28	0.00
C03/2	2668163.18	514696.31	2408.65	379907.50	367217.63	0.00
C03/3	3375548.55	186482.71	12636.00	548049.57	678856.47	0.00
C04/1	722947.60	195171.86	330.61	232319.29	125153.21	0.00
C04/2	1011436.68	255228.10	221.90	341438.29	171981.86	0.00
C04/3	2672234.13	339007.54	180.30	832030.13	692249.05	0.00
C04/4	2922631.37	352989.37	156.28	677275.84	505613.90	0.00
C05/1	1891206.07	727639.13	645.83	192574.41	262543.67	0.00
C05/2	2065267.30	671772.98	1507.87	210675.60	262463.98	0.00
C05/3	1733796.13	407584.71	443.34	214689.01	315715.33	0.00
C06/1	1570219.06	340733.04	445.59	390508.84	267016.08	0.00
C06/2	1072172.78	197189.12	120.55	251854.25	195722.06	0.00
C06/3	620482.19	152876.33	11.53	127675.45	117185.34	0.00
C07/1	357716.16	52897.34	84.70	112096.96	49725.20	0.00
C07/2	658745.28	71243.97	339.56	244238.66	95599.67	0.00
C07/3	767529.41	77325.31	314.44	283491.62	106300.82	0.00
C08	67127580.45	21630083.97	1104596.65	3016391.93	22372916.47	0.00
C09	126948085.08	19212984.02	2462247.54	9444500.94	68718443.26	0.00
C10	65278056.00	25196457.64	472238.95	1610808.79	14988708.20	0.00
C11	22910236.96	3611549.33	121147.51	750523.91	6857928.70	0.00
C12	47074467.77	18138079.80	353519.38	1308964.14	12538810.79	0.00
C13	286856946.54	16354683.44	372591.26	43891468.17	192415995.00	0.00
C14	58912801.05	10278138.84	527253.76	8869928.30	37824150.42	0.00
C15	137945616.44	15770084.32	510575.47	34278520.13	5002288.46	0.00
C16	30738626.46	10320149.60	576296.72	2839348.81	3367532.31	1807.64
MW	28180962.59	4724006.25	210408.35	3629476.09	11924367.92	78.59
STAB	60220057.59	7858906.72	492707.95	9807799.56	36394525.65	376.92

Table 6: Absolute numbers of total bacteria and bacterial subgroups in healthy controls analyzed by TaqMan-qPCR related to 10ng DNA

Patients	All bacteria	*Bacteroides*	Bifidobacteria	*C.* cluster *IV*	*C.* cluster *XIVa*	*C. difficile*
P01/1	1798805.94	686436.18	22965.45	431283.01	179653.46	0.00
P01/2	372479.33	214081.92	4558.69	66859.19	17518.96	0.00
P01/3	1183463.77	453079.56	30777.49	349670.05	81729.38	0.00
P01/4	4900917.36	1757221.14	69416.26	894221.86	492005.88	0.00
P02/1	20212193.78	5354904.16	25.84	1727067.70	8456716.97	0.00
P02/2	23903696.36	4005111.16	366.48	1926246.84	6273075.75	0.00
P02/3	3129907.77	1537813.68	65.15	899451.34	202334.40	0.00
P02/4	42134076.47	8601362.67	266.24	6391142.51	11186629.09	0.00
P03/1	5252430.48	330458.04	303812.95	1584639.87	703331.31	0.00
P03/2	32539656.72	7049121.86	1322331.64	11554656.05	10912078.21	0.00
P03/3	8776038.82	1914216.02	68520.97	1613986.64	3585565.52	0.00
P03/4	34133421.73	5633416.81	368271.38	6935425.53	17973732.55	0.00
P04/1	10176085.75	3675199.65	123237.25	2790373.95	1828490.92	0.00
P04/2	2041417.98	124427.95	6748.31	1044114.49	456546.61	0.00
P04/3	9397403.84	2436596.74	150717.25	2484676.03	1113862.60	0.00
P04/4	17611326.10	3143896.40	388478.41	5200829.47	1762498.85	0.00
P05/1	58606024.90	7574676.76	1485218.67	8089718.24	10498104.44	0.00
P05/2	77914671.15	9930958.08	4295028.01	24053291.85	25121947.29	0.00
P05/3	47867203.61	10669686.99	2232338.50	8288562.23	21327802.26	0.00
P05/4	45546066.19	8990544.45	1150309.59	11197726.83	23100668.54	0.00
P06/1	4254777.75	1117961.90	150748.62	648065.08	1921015.97	0.00
P06/2	19663552.55	4064824.71	143528.56	1769516.54	4984825.07	0.00
P06/3	5817626.36	1692997.14	126902.02	1911000.59	1832499.43	0.00
P06/4	11491330.22	2619686.08	354166.89	1450849.53	3690397.55	0.00
P07/1	32882071.40	13715080.97	897.46	3410583.24	7731920.78	0.00
P07/2	14010666.45	2027428.84	1032.00	2671080.63	1991983.19	0.00
P07/3	31475072.44	16519292.63	823.50	1586561.05	13562.81	0.00
P07/4	210972.37	82379.99	34.45	55314.07	9572.75	0.00
P08/1	20526835.88	5611144.84	718.75	1247117.30	7680688.02	0.00
P08/2	44425634.50	16318239.73	895.78	3476321.68	15493685.21	0.00
P08/3	25709141.44	11964895.51	718.60	2268109.88	5119868.83	0.00
P08/4	4789406.38	1833583.83	50.22	255512.39	404272.01	0.00
P09/1	60928403.72	19783140.36	349975.84	6483641.99	5835644.77	6882.51
P09/2	79557247.54	24331906.09	426303.13	7125594.64	6230655.43	3410.13
P09/3	18692507.80	7732250.88	2051.63	1534255.90	6614636.98	228486.37
P09/4	22422312.29	7987608.92	13249.55	1508579.53	2774425.14	90328.31
P10/1	13477917.70	2837015.05	73.34	1122511.68	1498517.07	0.00
P10/2	18520677.78	2213914.71	15.13	830143.08	993439.98	0.00
P10/3	52591368.06	5899979.48	3869.15	2670649.71	4623410.70	0.00
P10/4	21962667.14	5147834.94	1465.47	1149697.73	2797644.59	0.00

Table 7A: Absolute numbers of total bacteria and bacterial subgroups in the oncology patients analyzed by TaqMan-qPCR related to 10ng DNA, P01-P10

Patients	All bacteria	*Bacteroides*	Bifidobacteria	*C.* cluster *IV*	*C.* cluster *XIVa*	*C. difficile*
P11/1	44187524.40	6037882.96	441875.24	2509998.68	2374357.37	0.00
P11/2	9505774.72	865600.46	95057.75	1175348.89	2030535.80	0.00
P11/3	4652268.06	1640708.80	0.05	180029.67	397823.15	181438.45
P11/4	30489412.48	5277219.74	0.29	32637.34	292051.98	243915.30
P12/1	1101929.58	353588.40	1.37	111339.84	126430.54	0.00
P12/2	6715972.20	1220407.85	4.04	681879.34	730713.08	0.00
P12/3	2775027.99	721257.99	173.18	286183.85	509768.55	0.00
P13/1	1417002.07	236089.42	605.15	238326.79	210343.70	0.00
P13/2	1743935.23	229234.11	192.82	380815.57	219777.32	0.00
P13/3	9419678.45	1437380.19	1674.77	1821573.55	1187587.57	0.00
P13/4	2220094.29	214693.75	1193.98	383891.97	290244.10	3.95
P14/1	2812806.45	339245.97	51.39	619589.76	388784.99	82.69
P14/2	1898857.79	318576.67	226.39	440094.63	234728.66	0.00
P14/3	216491.56	100645.68	332.65	61728.96	31497.30	0.00
P15/1	819799.59	290536.62	344.12	148236.56	142420.79	0.00
P15/2	515193.86	148824.80	902.84	116214.55	84706.60	1.00
P15/3	809754.51	384999.39	5732.25	130018.93	130299.63	0.00
P15/4	1534925.19	486963.46	1752.75	175493.57	195136.85	0.00
P16/1	1378646.12	519302.17	19.94	209558.66	182349.69	6.97
P16/2	1343463.89	445758.10	7.04	200913.16	147572.01	0.00
P16/3	1152274.79	309769.22	99.83	170553.86	150962.71	0.00
P16/4	1229934.96	302951.10	48.50	243413.25	165421.24	0.00
P17/1	1221647.49	231511.65	2.50	151162.87	178143.59	0.00
P17/2	661624.52	71960.51	5.85	92751.67	128249.19	0.00
P17/3	2197256.20	267220.69	80.13	254390.04	369677.36	0.00
P17/4	1698079.84	315570.29	64.88	196263.35	248026.25	0.00
MW P01-17	16433740.21	3944731.47	214415.52	2298658.47	3616129.84	26948.42
STAB P01-17	19782724.05	5196061.66	642056.52	3798481.66	5779273.42	70054.14

Table 8B: Absolute numbers of total bacteria and bacterial subgroups in the oncology patients analyzed by TaqMan-qPCR related to 10ng DNA, P11-P17

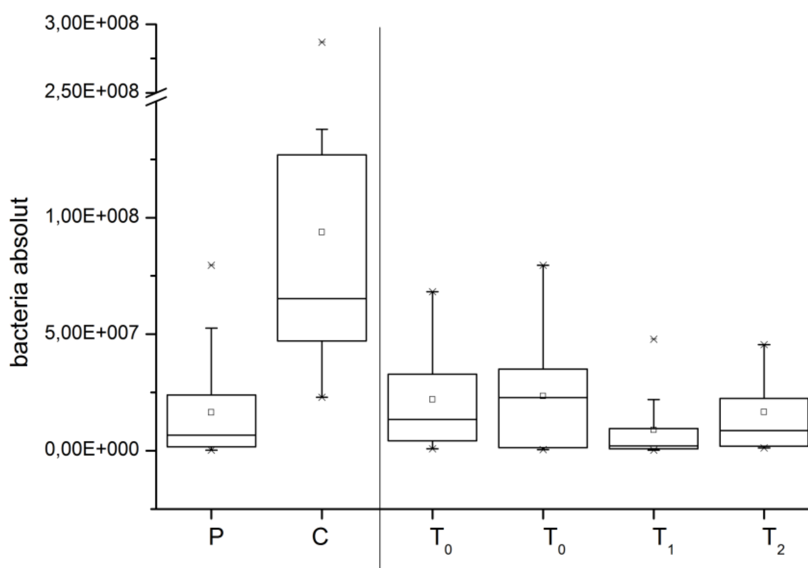

Figure 7: Comparison of absolute numbers of bacteria analyzed by TaqMan-qPCR in oncology patients and healthy controls and different time points of cycles of chemotherapy in copies/10ng DNA

P, oncology patients; C, healthy controls; T_o, before chemotherapy; T_1, 1-2d after chemotherapy, T_2 >2d after chemotherapy

As shown in figure 7, patients receiving chemotherapy (± antibiotics) harbored significantly less bacteria ($p< 0.05$) than healthy controls. Considerable differences were also shown among absolute numbers of microbiota of several sampling times: Absolute numbers of bacteria in different points in time of healthy controls are following a lognormal distribution in contrast to the shifts in oncology patients. Faecal microbiota of patients with cycles of chemotherapy decreased significantly more between two time points (p=0,027) than healthy controls.

As seen in figure 7 absolute numbers of bacteria ($p = 0.037$) were significantly lower directly after cycles of chemotherapy (T_1). There was also a significant decrease in the bacterial subgroups of *Bacteroides* (p=0.044), bifidobacteria (p=0.034) and *Clostridium* cluster *IV* (p=0.049). *Clostridium* cluster *XIVa* was

effected too, but not significantly. Figure 8 illustrates that all patients with fever (P01/4, P08/2, P09/2) showed an increase of total fecal microbiota. Sample P07 showed at time point 4 a sharp decline affecting all bacteria and bacterial subgroups after a blood stem cell transplantation. After the end of

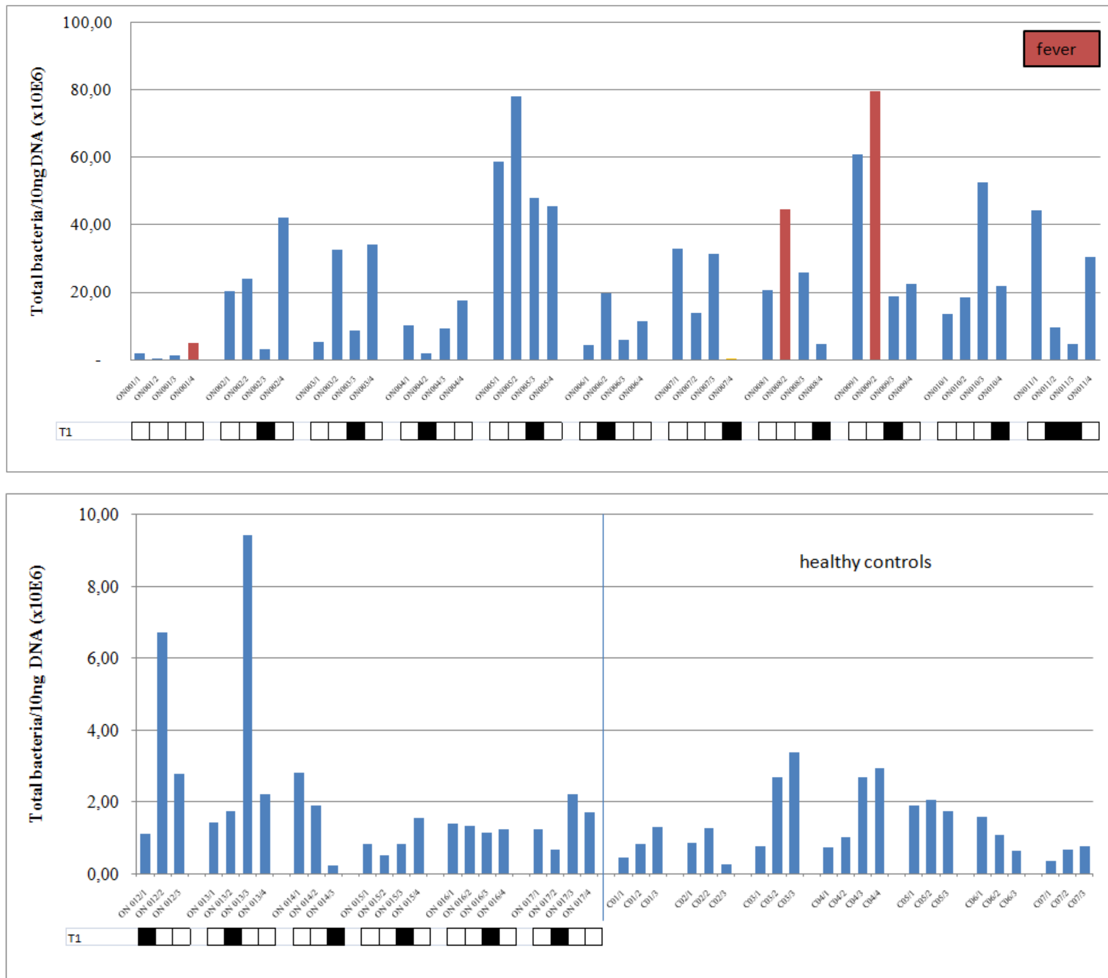

Figure 8: Absolute numbers of bacteria of the study population analyzed by TaqMan-qPCR related to 10ng DNA; T_1, 1-2d after chemotherapy

chemotherapeutic treatment the abundance of fecal microbiota went back to normal levels within a few days. Six patients even showed a “rebound effect” within the recovery phase with a short lived increase of total bacteria to higher amounts than in the beginning.

9.2.2. Relative quantification of bacterial subgroups

Table 8 and figure 9 show the quantification of bacterial subgroups as percentage of total bacterial DNA at the different points in time. Chemotherapy patients had a mean percentage of 26 ± 12% *Bacteroides*, while healthy controls harbored 22 ± 14%. Patients harbored in average 0.8 ± 1.4 % bifidobacteria and controls 0.3 ± 0.6 %. In patients the mean proportion of *Clostridium* cluster *IV* and *Clostridium* cluster *XIVa* was 16 ± 9 % and 19 ± 12 %, while controls harbored on average 20 ± 12 % and 24 ± 15 %.

Patients	Bac.%	Bif.%	C.Cl.IV%	C.Cl.XIVa%	C.diff.%	unidentified%
P01/1	38,161	1,277	23,976	9,987	0,000	26,599
P01/2	57,475	1,224	17,950	4,703	0,000	18,648
P01/3	38,284	2,601	29,546	6,906	0,000	22,663
P01/4	35,855	1,416	18,246	10,039	0,000	34,444
P02/1	26,493	0,000	8,545	41,840	0,000	23,122
P02/2	16,755	0,002	8,058	26,243	0,000	48,942
P02/3	49,133	0,002	28,737	6,465	0,000	15,663
P02/4	20,414	0,001	15,169	26,550	0,000	37,866
P03/1	6,292	5,784	30,170	13,391	0,000	44,364
P03/2	21,663	4,064	35,509	33,535	0,000	5,229
P03/3	21,812	0,781	18,391	40,856	0,000	18,160
P03/4	16,504	1,079	20,319	52,657	0,000	9,441
P04/1	36,116	1,211	27,421	17,969	0,000	17,284
P04/2	6,095	0,331	51,147	22,364	0,000	20,064
P04/3	25,928	1,604	26,440	11,853	0,000	34,175
P04/4	17,852	2,206	29,531	10,008	0,000	40,404
P05/1	12,925	2,534	13,804	17,913	0,000	52,824
P05/2	12,746	5,512	30,871	32,243	0,000	18,627
P05/3	22,290	4,664	17,316	44,556	0,000	11,174
P05/4	19,739	2,526	24,585	50,719	0,000	2,430
P06/1	26,275	3,543	15,231	45,150	0,000	9,800
P06/2	20,672	0,730	8,999	25,351	0,000	44,249
P06/3	29,101	2,181	32,848	31,499	0,000	4,370
P06/4	22,797	3,082	12,626	32,115	0,000	29,381
P07/1	41,710	0,003	10,372	23,514	0,000	24,401
P07/2	14,471	0,007	19,065	14,218	0,000	52,240
P07/3	52,484	0,003	5,041	0,043	0,000	42,430
P07/4	39,048	0,016	26,219	4,537	0,000	30,180
P08/1	27,336	0,004	6,076	37,418	0,000	29,168
P08/2	36,732	0,002	7,825	34,876	0,000	20,566
P08/3	46,539	0,003	8,822	19,915	0,000	24,721
P08/4	38,284	0,001	5,335	8,441	0,000	47,939
P09/1	32,469	0,574	10,641	9,578	0,011	46,726
P09/2	30,584	0,536	8,957	7,832	0,004	52,087
P09/3	41,366	0,011	8,208	35,387	1,222	13,807
P09/4	35,623	0,059	6,728	12,374	0,403	44,813
P10/1	21,049	0,001	8,329	11,118	0,000	59,503
P10/2	11,954	0,000	4,482	5,364	0,000	78,200
P10/3	11,219	0,007	5,078	8,791	0,000	74,905
P10/4	23,439	0,007	5,235	12,738	0,000	58,581
P11/1	13,664	1,000	5,680	5,373	0,000	74,282
P11/2	9,106	1,000	12,365	21,361	0,000	56,168
P11/3	35,267	0,000	3,870	8,551	3,900	48,412
P11/4	17,308	0,000	0,107	0,958	0,800	80,827
P12/1	32,088	0,000	10,104	11,474	0,000	46,334
P12/2	18,172	0,000	10,153	10,880	0,000	60,795
P12/3	25,991	0,006	10,313	18,370	0,000	45,320
P13/1	16,661	0,043	16,819	14,844	0,000	51,633
P13/2	13,145	0,011	21,837	12,602	0,000	52,405
P13/3	15,259	0,018	19,338	12,608	0,000	52,777
P13/4	9,670	0,054	17,292	13,074	0,000	59,910
P14/1	12,061	0,002	22,027	13,822	0,003	52,085
P14/2	16,777	0,012	23,177	12,362	0,000	47,672
P14/3	46,489	0,154	28,513	14,549	0,000	10,295
P15/1	35,440	0,042	18,082	17,373	0,000	29,063
P15/2	28,887	0,175	22,557	16,442	0,000	31,938
P15/3	47,545	0,708	16,057	16,091	0,000	19,599
P15/4	31,726	0,114	11,433	12,713	0,000	44,014
P16/1	37,668	0,001	15,200	13,227	0,001	33,903
P16/2	33,180	0,001	14,955	10,984	0,000	40,880
P16/3	26,883	0,009	14,801	13,101	0,000	45,205
P16/4	24,631	0,004	19,791	13,450	0,000	42,124
P17/1	18,951	0,000	12,374	14,582	0,000	54,093
P17/2	10,876	0,001	14,019	19,384	0,000	55,720
P17/3	12,162	0,004	11,578	16,824	0,000	59,433
P17/4	18,584	0,004	11,558	14,606	0,000	55,248
MW	**25,97**	**0,80**	**16,30**	**18,34**	**0,10**	**38,49**
STAB	**12,21**	**1,39**	**9,40**	**12,17**	**0,51**	**19,01**

Controls	Bac.%	Bif.%	C.Cl.IV%	C.Cl.XIVa%	C.diff.%	unidentified%
C01/1	13,981	0,056	30,643	17,716	0,000	37,604
C01/2	6,414	0,055	38,822	27,566	0,000	27,142
C01/3	9,621	0,050	42,803	30,686	0,000	16,841
C02/1	24,193	0,000	15,873	26,079	0,000	33,854
C02/2	25,088	0,000	13,398	22,895	0,000	38,619
C02/3	25,498	0,000	13,014	30,415	0,000	31,073
C03/1	74,207	0,132	14,831	7,639	0,000	3,191
C03/2	19,290	0,090	14,239	13,763	0,000	52,618
C03/3	5,525	0,374	16,236	20,111	0,000	57,754
C04/1	26,997	0,046	32,135	17,312	0,000	23,511
C04/2	25,234	0,022	33,758	17,004	0,000	23,982
C04/3	12,686	0,007	31,136	25,905	0,000	30,266
C04/4	12,078	0,005	23,173	17,300	0,000	47,443
C05/1	38,475	0,034	10,183	13,882	0,000	37,426
C05/2	32,527	0,073	10,201	12,708	0,000	44,490
C05/3	23,508	0,026	12,383	18,209	0,000	45,874
C06/1	21,700	0,028	24,870	17,005	0,000	36,397
C06/2	18,392	0,011	23,490	18,255	0,000	39,852
C06/3	24,638	0,002	20,577	18,886	0,000	35,897
C07/1	14,788	0,024	31,337	13,901	0,000	39,951
C07/2	10,815	0,052	37,076	14,512	0,000	37,545
C07/3	10,075	0,041	36,936	13,850	0,000	39,099
C08	32,222	1,646	4,494	33,329	0,000	28,310
C09	15,135	1,940	7,440	54,131	0,000	21,355
C10	38,599	0,723	2,468	22,961	0,000	35,249
C11	15,764	0,529	3,276	29,934	0,000	50,497
C12	38,531	0,751	2,781	26,636	0,000	31,302
C13	5,701	0,130	15,301	67,077	0,000	11,791
C14	17,446	0,895	15,056	64,204	0,000	2,399
C15	11,432	0,370	24,849	3,626	0,000	59,722
C16	33,574	1,875	9,237	10,955	0,006	44,353
MW	***22,069***	***0,322***	***19,742***	***23,499***	***0,000***	***34,368***
STAB	***13,793***	***0,556***	***11,692***	***14,637***	***0,001***	***13,856***

Abbreviation	Meaning
Bac.%	Bacteroides %
Bif.%	Bifidobacteria %
C.Cl.IV%	Clostridium Cluster IV %
C.Cl.XIVa%	Clostridium Cluster XIVa %
C.diff.%	Clostridum difficile %
unidentified%	unidentified species &

Table 9: Relative numbers of bacterial subgroups

As illustrated in figure 10 patients under chemotherapeutical administration showed significantly less *Clostridium* cluster *XIVa* (p = 0,021) than healthy individuals. The other observed subgroups showed no statistically significant difference between oncology patients and healthy subjects.

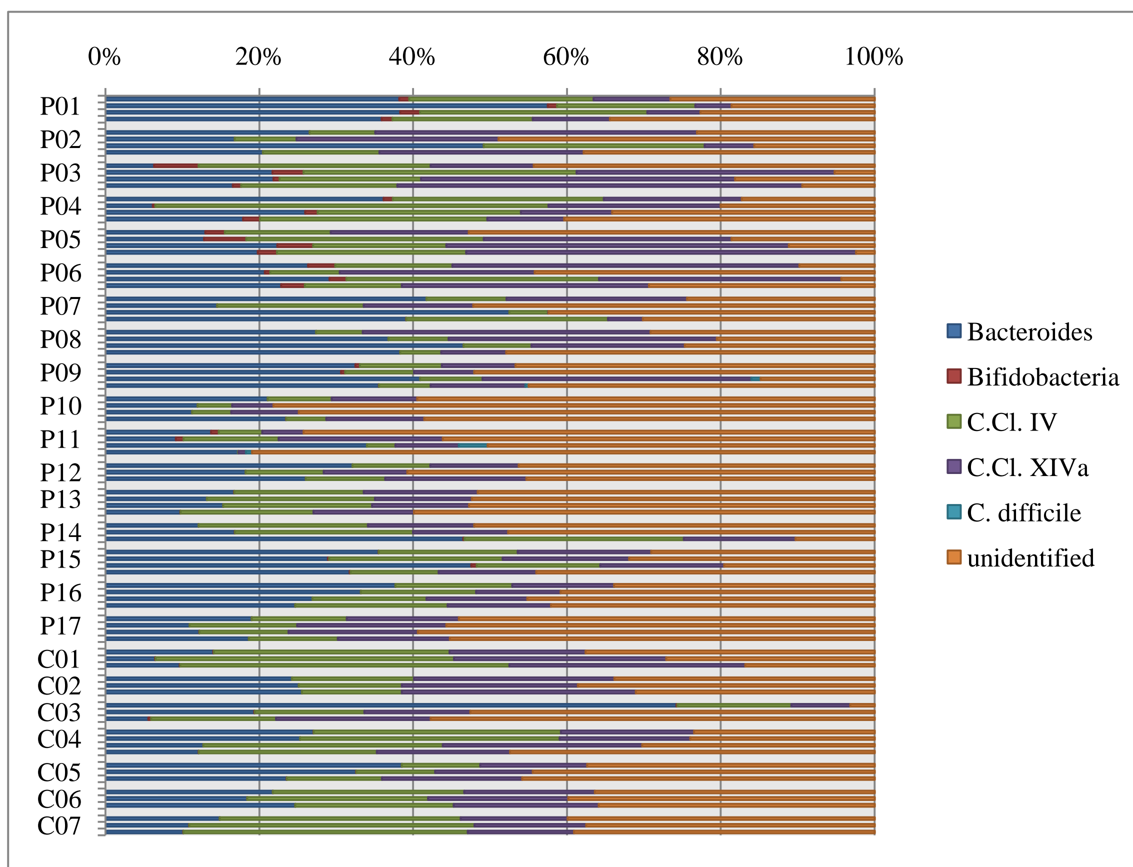

Figure 9: Percentages of bacterial subgroups in relation to analyzed bacteria

Figure 10 shows that the mean percentage of *Clostridium* cluster *XIVa* decreased after bouts of chemotherapy. Before chemotherapy the mean proportion of *Clostridium* cluster *XIVa* was 22 ± 13% compared to 19 ± 12% after chemotherapeutical treatments.

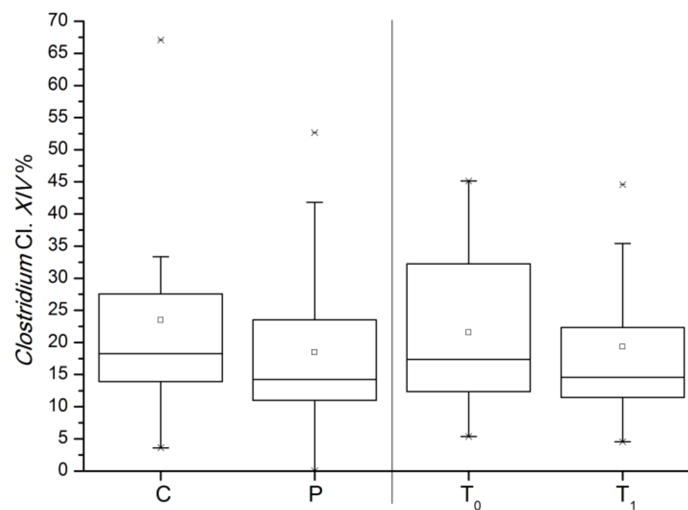

Figure 10: Relative abundances of *Clostridium* Cluster *XIVa* in oncology patients compared to healthy controls and before and after chemotherapeutic cycles as percentage of total bacterial DNA. P, oncology patients; C, healthy controls; T_o, before chemotherapy; T_1, 1-2d after chemotherapy

Oncology patients harbored 26 ± 11%, 1.4 ± 2% and 16 ± 9% of *Bacteroides*, bifidobacteria and *Clostridium* cluster *IV* before chemotherapy and 28 ± 14%, 0.5 ± 1.2% and 18 ± 12 % after cycles of chemotherapy. The differences were not significant.

We tried to find out whether a chemotherapeutic and/or antibiotic disruption of the microbiota favors the growth of pathogens by investigating all samples for pathogenic *Clostridium difficile.* In three out of seventeen patients (18%) receiving chemotherapeutic ± antibiotic treatment *Clostridium difficile* was found (P09, P11, P14), especially subject P09 and P11 harbored considerable numbers. Patient P09 harbored *C. difficile* in all four samples investigated. The mean percentage of *C. difficile* in this subject over all four points in time was 0.4 ± 0.7%, the highest number was recorded at time point T1 (P09/3), which was taken immediately after a bout of chemotherapy and a treatment with antibiotics. Also in patient 11 and patient 14 a colonization with *C. difficile* was

found after chemotherapeutic intervention. (P11: 3,90%, P14: 0,003% of all analzyed bacteria). Sample P09/1, P09/3, P11/1 and P11/3 at time points T0 and T1 of patients P9 and P11 were further analyzed by 454 sequencing.
Small amounts of *C. difficile* were also found in three other patients, but they were below the detection limit. Except for C16 with a proportion of 0,006% of all bacteria, healthy controls didn't harbor *C. difficile*.

9.3. DGGE bandpattern analysis

DGGE fingerprinting analyses of total bacteria, *Clostridium* cluster *IV* and *Clostridium* cluster *XIVa* point out a high diversity of fecal microbiota between individuals. Table 9 shows the mean number of bands per oncology patient and healthy control.

Oncology patients

Bacterial group	**Mean number of bands**
All bacteria	19 ± 4
Clostridium cluster *IV*	14 ± 6
Clostridium cluster *XIVa*	14.9 ± 7

Healthy controls

Bacterial group	**Mean number of bands**
All bacteria	19 ± 3.5
Clostridium cluster *IV*	12 ± 5
Clostridium cluster *XIVa*	18.9 ± 7

Table 10: Mean number of bands in study population analyzed with PCR-DGGE and GelCompareII

The number of *Clostridium* cluster *IV* bands before chemotherapy (T0) was 14 ± 7, whilst immediately after chemotherapy (T1) the number declined to 10 ± 6. At time point T2 meaning a few days after chemotherapy the number of bands increased to 15 ± 6. The dataset was subjected to principal component analysis (PCA), which extracts underlying components of samples according to their variance.

Figure 11 A displays the bacterial fingerprints of sample P01 over the course of time. Figure 11 B illustrates the Shannon diversity index of *Clostridium* cluster *IV* fingerprints, indicating characteristics before and after chemotherapy in oncology patients compared with the healthy control group. DGGE fingerprints

of individuals show smaller variability in PCA analysis after chemotherapy (T1 and T2) compared to healthy controls (C) and patients before onset of treatment (T0). PCA resulted in distinctive clustering of band patterns before and after chemotherapy [ZWIELEHNER, 2010].

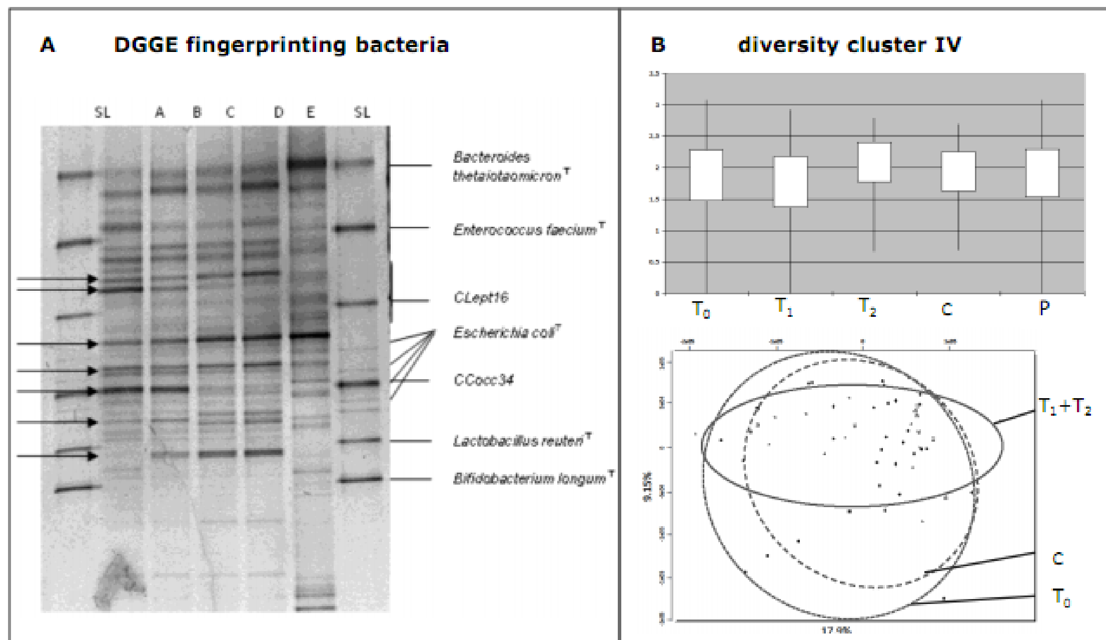

Figure 11A: Changes of PCR-DGGE fingerprinting of 16S rRNA coding regions of dominant bacteria after chemotherapeutic treatment. Arrows indicate nearly disappearing or stronger bands. A, B, C and D, samples of ON001 after chemotherapy; E, healthy control; SL, standard lane

11B: Shannon diversity index of *Clostridium* cluster *IV* fingerprinting and PCA [ZWIELEHNER, 2010]

9.4. High throughput sequencing

As shown in figure 12 454 sequencing showed a severe decrease of *Faecalibacterium spp.* as well as lactobacilli, *Veillonella spp.,* bifidobacteria (in P09) and *E.coli / Shigella* immediately after chemotherapy in oncology patients P09 and P11.

P09 additionally received concomitant antibiotics. Especially *Faecalibacterium spp.* was strongly reduced from 9.5% and 8.3% to 0.07% and 0.00%,

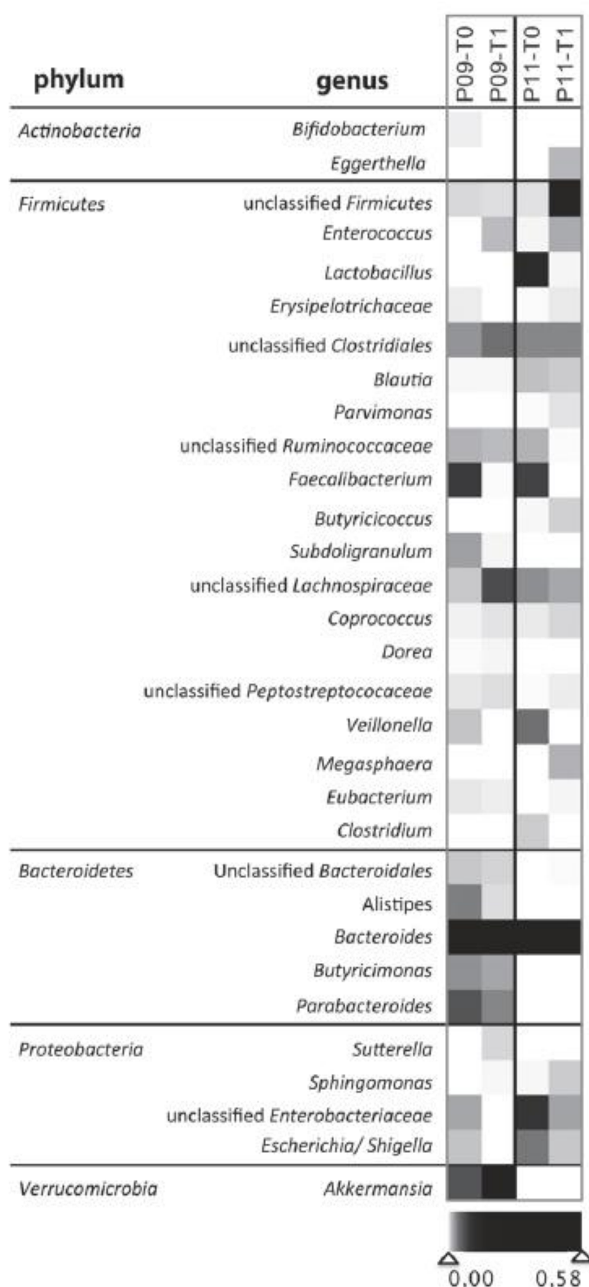

Figure 12: Heatmap showing abundances within the 454 sequencing dataset on the genus level. Species with abundance <0.01% of all sequences are not shown. [ZWIELEHNER et al., 2011]

respectively. In contrast *Enterococcus faecium* increased after chemotherapy in both individuals. Furthermore, a strong increase in sequences within the *Peptostreptococcaceae* towards sequences 98.9–100% similar to *C.difficile*^{T} occurred: *Clostridium bartletti* related sequences (98.1–100% similarity) were only detected in fecal samples taken before chemotherapy (T0). Immediately after chemotherapy (T1), 63 sequences 98.9–100% similar to *C.difficile* appeared in samples P11 and P09, they are shown as 'unclassified *Peptostrepto-coccaceae*' in figure 12.

Further less abundant sequences appeared that were not detected before treatment: *Eggerthella, Megasphaera, Parvimonas, Anaerostipes, Eubacterium, Anaerococcus, Methylobacterium, Holdemania, Turicibacter, Akkermansia, Sutterella* (in P09), *Sphingomonas, Anaerotruncus, Coprococcus, Streptococcus* and *Dorea.* The number of *Blautia* species in *C.* cluster *XIVa* remained constant before and after chemotherapeutic treatment.

10. DISCUSSION

Chemotherapeutic regimen and administration of antibiotics cause severe side effects in oncology patients, which often result in life threatening complications and increase the cost of health services. Diarrhea and constipation are common harmful secondary effects, furthermore mucositis is a major problem induced by the cytotoxic effects of cancer therapy and radiotherapy [GIBSON et al., 2006; STRINGER et al., 2007]. Chemotherapeutic and antibiotic use increases the risk of infection and overgrowth with potential pathogenic species such as *C. difficile* by interfering with the composition of the native gut microbiota [VAN VLIET et al. 2009].

In this study we investigated how the gastrointestinal microbiota of oncology patients was affected during cycles of chemotherapy (± antibiotic administration). We evaluated the shifts of the microbial ecosystem following chemotherapy and antiobiotic treatment and tried to assess if the microbiota was able to recover fully after therapy. Special focus was on the abundance on potential pathogenic *C. difficile*.

10.1. Material and Methods

The majority of the **study population** had a longer history of different chemotherapy treatment regimes over the course of several years. Only two study participants (P01 and P08) had never received any cancer therapy before. The results in this study illustrate changes due to a single bout of chemotherapy, but the observed shifts in microbiota are likely to be affected by previous cycles. For this reason, the results cannot rule out, that observed changes are influenced by previous therapies. For example, cancer patients showed a significantly lower abundance of total bacteria numbers, which could be the result of previous treatments.

Furthermore the treatments were different and they suffered from a variety of malignancies. Six oncology patients also received antibiotics, which poses the risk to falsely interpret the effects of antibiotic treatment as effects of

chemotherapy. However, they showed a similar response to chemotherapy like the rest of the study group, so it can be hypothesized that the disbalance of the gut microbiota was caused by the chemotherapeutic intervention itself. Three patients (P12, P13 and P17) suffered from different kinds of cancer of the intestine. A recent work observed a composition change in the microbiota of colon cancer patients [SOBHANI et al., 2011]. This fact should be considered in future investigations.

The human gastrointestinal tract is colonized by autochthonous microbiota [WANG et al., 2003]. The bacterial communities found at different sites of the intestine vary significantly, especially when comparing samples of upper GI-tract with samples from the lower intestine [PASTER et al., 2001; PEI et al., 2004). It was postulated in previous works that the fecal microbiota consists of shed mucosal bacteria and a separate nonadherent luminal population [ECKBURG et al., 2005]. While further studies suggested that fecal samples do not necessarily represent the microbial populations in other parts of the gastrointestinal tract [WANG et al., 2003; ZOETENDAL et al., 2002], a recent work of Van der Waaij et al. showed that the composition of the bacterial microbiota present in the feces was similar to that at the mucus layer of the terminal ileum and colon regions. Furthermore they did not observe direct contact between epithelial cells and bacteria, which suggested that there is no specific mucus-adherent microbiota [VAN DER WAAIJ et al., 2005].
Due to the inaccessibility of human intestinal tract we chose fecal samples for our investigations, even though the fecal microbiota might differ from the adherent microbiota, which can only be assessed by colonic biopsies. Compared to biopsies, fecal samples are easy to collect, less invasive and do reflect changes in microbial population composition of the gut [TURRONI et al., 2008].
Stool samples were frozen prior to DNA extraction, which is widely used in metagenomic studies. However, a recent work observed that freezing may affect the community composition. In this study the *Firmicutes* to *Bacteroidetes*

16S rRNA gene ratio was significantly higher in stool samples that had been frozen compared to identical samples that had not been [BAHL et al., 2012].

Previous works have done research on the effects of chemotherapeutic treatment ± antibiotic use on the intestinal microbiota [VAN DER WAAIJ, 1984; STRINGER et al., 2008]. The majority of these earlier studies applied culture dependent methods, which were common practice before the development of molecular detection methods. Fecal microbiota was monitored and identified mainly by cultivation on selective growth medium and other culture dependent methods, which are limited in assessing entire microbial populations because a large fraction of gastrointestinal bacteria has never been cultured and escapes detection. The implementation of culture-independent approaches to study the microbial gut composition has made it possible to assess the host's intestinal microbiota independent of culturing techniques and thus, broadened our knowledge of the diversity of species present in the gastrointestinal microbiota [MUYZER et al. 1993, MUYZER, 1999]. The investigation of rRNA as a molecular clock has become established for phylogeny and classification of microbes and the 16SrRNA gene harbours sufficient variable region of identification and differentiation of specific species [ZOETENDAL et al., 2006].

In this study a combination of **16S rRNA based molecular methods** was applied. Real time quantitative PCR was used for relative and absolute quantification of all bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV*, *Clostridium* cluster *XIVa* and *Clostridium difficile*. Although PCR is a highly sensitive and specific technique, all PCR-based methods are affected by bias, induced among others by rehybridisation of PCR products or different primer binding energy. To ensure an accurate and successful qPCR assay, in this work specificity of all PCR reactions was confirmed using target and non-target DNA and sensitivity of all PCR reactions was determined with 10 fold dilutions of standard curve DNA. Furthermore, clone libraries were constructed and the obtained sequences allowed an estimate of the primer specificity.

Species richness and diversity was assessed using **PCR-DGGE fingerprinting**. Each lane of the PCR-DGGE gel displays a microbial fingerprint of a stool sample; within a lane each band usually represent one bacterial species, although it also happens that different species are represented by the same band [VAN VLIET et al., 2009] or one bacterial strain form several bands. Latter is associated with species including multiple 16S rRNA operons, e.g. *E.coli*. Limitations of DGGE technique in analysis microbial communities have been previously described [MUYZER et. al, 1998].

DNA sequencing is used for determining the DNA sequence meaning the order of the nucleotides bases (A, G, C, T) in a DNA molecule. Demand for DNA sequence information has never been greater, since earlier commonly used Sanger technology is too costly, time consuming, and labor intensive to meet this ongoing demand [METZKER, 2005]. There is a coming of several new high-throughput technologies, all having in common the demand for low cost sequencing with parallelization of the sequencing process in order to produce thousands or millions of sequences at once. Pyrosequencing itself is no high throughput sequencing technology but provides the basis for 454 sequencing. In contrast to Sanger sequencing which relies on chain termination with dideoxynucleotides pyrosequencing is based on the detection of pyrophosphate release during DNA synthesis. High-throughput molecular technologies can profile microbial populations at high resolution even in complex environments like the gastrointestinal tract. To gain a decent accuracy it is important to minimize the amplification bias and to select sequencing primers and variable 16S rRNA gene regions with great care [CLAESSON et al., 2010].

10.2. Results

The results show significant effects of chemotherapy and antibiotic treatment on the intestinal microbiota. The absolute abundance of bacteria was significantly lower in patients under chemotherapeutic administration than in healthy controls, which is in agreement with previous studies [VAN VLIET et al., 2009]. The numbers of bacterial subgroups changed together with total bacteria both in

patients with and without antibiotics. To consider the high individual variations both test and healthy control group were observed over a course of time, thus significant differences were also shown among absolute numbers of microbiota of several sampling times: the total bacteria load in different points of time in healthy controls followed a lognormal distribution in contrast to the shifts in oncology patients. Fecal microbiota of patients with cycles of chemotherapy decreased significantly more between two time points than healthy controls. Furthermore, numbers of total bacteria, bifidobacteria, *Bacteroides*, and *Clostridium* cluster *IV* were significantly lower directly after cycles of chemotherapy (T_1). In contrast, increased relative counts of *Bacteroides spp*. could be observed in patients undergoing chemotherapeutical treatment, which is in agreement with previous results [NYHLEN et al., 2002]. Nyhlen et al. also reported increased counts of yeasts in patients, but yeasts were not subject of this study.

After the end of chemotherapeutic treatment the abundance of fecal microbiota went back to normal levels within a few days. Six patients even showed a “rebound effect” within the recovery phase with a short lived increase of total bacteria to higher amounts than in the beginning. All patients with fever (P01/4, P08/2, P09/2) showed an increase of total fecal microbiota. Fever in immunocompromised patients is the principal and sometimes the only manifestation of serious infection [PIZZO, 1999], which might be due to increased numbers of pathogenic species in the gut or an overall bacteria overgrowth.

Species richness decreased immediately after a chemotherapeutic cycle. Especially *Clostridium* cluster *IV* was affected with a number of 14±6.7 different bands before chemotherapy (T0) decreasing to 10±6.6 bands shortly after (T1). Even after recovering to 15±6.6 bands later, PCA observed a persisting different composition of *Clostridium* cluster *IV* species.

PCR DGGE analysis revealed that no significant differences existed in fingerprints of all bacteria in patients with or without antibiotic treatment. *Clostridium* cluster *XIVa* showed a decreased number of bands, but differences were not significant. Results indicate that the observed changes might be either due to chemotherapy itself or additional effects. This finding is in line with data from literature showing additional effects of chemotherapy in cases under antibiotic treatment [VAN VLIET et al., 2009]. Abundance of *Clostridium* cluster *IV* did not differ significantly under antibiotic use, while the diversity did decrease. Principal component analysis of PCR DGGE fingerprints showed grouping of patients under antibiotic treatment.

High-throughput sequencing

Our qPCR results showed that the emergence of *C. difficile* in oncology patients P09 and P11 in point in time T01 immediately after chemotherapy went along with a decline of the genera *Bifidobacterium, Lactobacillus* and *Clostridium* cluster *IV*. These samples were of special interest for us and therefore submitted to 454 barcode sequencing. Results indicated a dramatically decrease of sequences attributable to *F. prausnitzii* from 9% to zero. *F. prausnitzii* is a commensal antiinflammatory bacterium of the human gut flora. Low levels of *F. prausnitzii* may be associated with Crohn's disease [SOKOL et al., 2008]. After chemotherapy facultativ pathogenic *Enterococcus faecium* increased and further less abundant sequences appeared that were not detected before treatment: *Eggerthella, Megasphaera, Parvimonas, Anaerostipes, Eubacterium, Anaerococcus, Methylobacterium, Holdemania, Turicibacter, Akkermansia, Sutterella, Sphingomonas, Anaerotruncus, Coprococcus, Streptococcus* and *Dorea*. Propionate producing *Megasphaera spp*. and butyrate-producers *Anaerostipes caccae* and *Eubacterium hallii* utilize lactat [FLYTHE et al., 2010; MARQUET et al., 2009]. Marquet et al. suggested that *A. caccae* and *E. hallii* compete for lactate with sulfate-reducing bacteria and high concentrations of sulfate in the gut epithelium may contribute to bowel disease. Microorganisms of the genus *Methylobacterium* are gram negative, facultative methylotrophic, and ubiquitous in nature. They rarely cause human

disease, mainly only in subjects with preexisting causes of immune depression [FANCI et al., 2010]. Fanci et al. decribed an unusual *Methylobacterium fujisawaense* infection in a patient with acute leukaemia undergoing hematopoietic stem cell transplantation. Poorly known *Turicibacter spp*. was previously found in weaned piglets and is known to be susceptible to the growth-promoting antibiotic chlortetracycline [RETTEDAL et al., 2009]. In humans the genus *Turicibacter* has been found in the ileal pelvic pouch of a former ulcerative colitis patient [FALK et al., 2007]. *Akkermansia muciniphila*, a anaerobic bacterium belonging to the *Verrucomicrobia*, is specialized in the degradation of mucin, the glycoprotein present in mucus, and is found in high numbers in the intestinal tract of human and other mammalian species. *A. muciniphila* is suggested to modulate pathways involved in establishing homeostasis for basal metabolism and immune tolerance toward commensal microbiota [DERRIEN et al., 2011]. *Dorea spp*. are members of the *Clostridium coccoides* rRNA group of organisms and are mucosa-associated bacteria of the human gastrointestinal tract [TARAS et al. 2002; NOMURAS et al., 2005].

Further research will have to show if the growth of *Clostridium difficile* is associated with the decline of *Lactobacillus spp., Bifidobacterium spp*. and *Clostridium cluster IV*, especially if there is a causal relationship to the decrease of *Faecalibacterium prausnitzii*. Also, the emergence of *C. difficile* might result in a increase of mucus degrading bacteria such as *Akkermansia muciniphila*. Mucus hypersecretion is a common symptom of bacterial infections of the gut epithelium, irritable bowel syndrome and ulcerative colitis. [GOODMAN et al., 1977; GUIMEAU et al., 2008].

In previous studies it has been shown that chemotherapeutic treatment is associated with a decrease in the microbial diversity and a decline of total bacterial counts. Moreover, research has shown that decreasing species richness coincides in time with the development of severe chemotherapy-induced mucositis [VAN VLIET et al., unpublished data]. We observed shifts in species composition especially in *Clostridium* cluster *IV*, additionally affected by

antibiotics. We conclude that antimicrobial treatment significantly reduces the diversity of *Clostridium* cluster *IV*. Further research is needed to observe possible effects of shifts in the gastrointestinal microbiota regarding infection, inflammation and the development and maintenance of mucosal barrier function. Further investigations will also have to show the clinical relevance of restoring dysbalances of the gut microbiota and thereby prevent mucosal barrier injury and its complications. Therefore prebiotic, probiotics or synbiotics could be used with the consideration for the risk of whole live bacteria used as probiotics causing invasive infections in immunocompromised patients.

In conclusion, chemotherapy treatment results in shifts in fecal microbiota, which come together with the emergence of *Clostridium difficile* in some patients. These changes affect and alter the functions of the GI microbiota and may contribute to severe side effects in oncology patients.

11. PROTOCOLS

11.1. DNA extraction from stool using QIAamp DNA Stool Mini Kit & Beadbeating

All centrifugation steps at max. speed. Prepare: ice. Keep samples always frozen. Work with fire and ethanol, spatula and scalpel should be sterilized between different samples. Prepare two heat-blocks at 95°C and 70°C. If a precipitate has formed in ASL buffer or AL buffer, solve in waterbath. Anyways vortex well before use.

1. Weight 180-220 mg frozen sample in a lysing matrix E (soil kit) tube.
2. Add 1.4 ml ASL buffer. Vortex well.
3. Secure lysing matrix tubes in beadbeater. Two cycles of 45 sec beadbeating with one intervening minute on ice.
4. Lysis for 5 min at 95°C
5. Vortex for 15 sec, centrifuge 1 min.
6. Pipet supernatant in a new 2 ml eppendorf tube. Discard pellet.
7. Add inhibitEX tablet. Vortex immediately until completely dissolved. Incubate 1 min at r/t
8. Centrifuge 6 min
9. Pipet supernatant into a clean 1.5 ml eppendorf tube. Discard pellet.
10. Centrifuge 3 min
11. Pipet 25µl proteinase K into a clean 2ml tube.
12. Add supernatant (approx 600µl)
13. Add 600 µl buffer AL. mind that proteinase K and buffer AL may never come directly into contact. Never change the order of steps 11-13.
14. Incubate 10 min at 70°C
15. Add 600 µl ethanol (96-100%) to the lysate and mix by vortex.
16. Label the lid of a new QIAamp spin column provided in a 2 ml collection tube.
17. Carefully apply 600 µl lysate from step 12 to the QIAamp spin column without moistening the rim. Centrifuge for 1 min, discard filtrate and place the spin column again in the 2 ml collection tube.
18. Repeat step 17 until all lysate has been used.
19. Carefully open the spin column and add 500 µl buffer AW1. Centrifuge 1 min.
20. Discard flow-through.
21. Carefully open the spin column and add 500 µl buffer AW2. Centrifuge 3 min.

22. To remove residual buffer AW2, place the spin column into a new collection tube and centrifuge for another 1 min
23. Transfer spin column into a new labelled 1.5 eppendorf tube. Elute with 200µl RNase, DNase free water, preheated to 80C. Centrifuge 1 min.
24. Elute a 2nd time with 200 µl $H2O$ (80C).

11.2. Cloning protocol

First two steps:

- Perform PCR/DGGE with several samples, thereafter choose the sample with the most diverse bandpattern
- Purify PCR products with QIAGEN PCR Purification Kit

First Day

---

1. **Prepare a ligation-reaction mixture** according to the following scheme with the control (included in Kit) and the purified sample: promega pGEM-T Easy Vector System I

7,5µl Buffer
+1µl Vector
+1µl Ligase
+5,5µl PCR pur. Or +3µl of control
15µl total volume → incubate at 4°C over night

2. **Prepare for the next day:**
   - Prepare 4 LB/Ampicillin/IPTG/X-Gal plates for each ligation reaction **+** 1 plate for determining transformation efficiency

   - **LB solid media:** Mix for 500ml LB-plating-media:

5g Tryptophan
2,5g Yeast Extract
2,5g NaCl
7,5g Agar
→ autoklav (1h)
At 50°C: +1ml Ampicilin (50mg/0,5ml)

   - **Pour plates**: 20ml per plate

  - Work under a Bunsen burner without gloves with disinfected hands
  - Avoid air bubbles in the agar by swiveling
  - Let the plates dry over night, thereafter pile upside down in a plastic bag

- **LB liquid media 100ml**

1g Trypton
0,5g Yeast extract
0,5g NaCl
Fill up with H20 dest. to 100ml and stir
➔ Autoklav 1h

Second Day

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1. **Transformation:** promega JM109 competent cells (200ml aliquots):
   - Transfer 10µl of the ligation to 100µl competent cells on ice for 1h
   - Heat-Shock: 42°C for 90sec, and immediately 5min on ice. Do not shake!
   - Add 500µl LB media on the competent cells and incubate at 37°C 1h with shaking 150rpm
   - Mix the tubes with your snapping finger

   Always work on ice and with cut off pipette tips with competent cells!

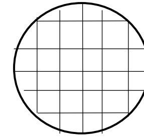
2. **Plates:**
   - Plate 20µl -IPTG/ X-Gal
   - Plate 100µl of each transformation culture; (2 plates with each100µl), plate control just once
   - Dilute and mix the rest with 100µl LB-Media
   - Plate 2 x100µl of each deluted transformation culture
   - Incubate at 37° over night (Incubator)

Third Day

---

1. **Screening for blue and white colonies**
   - Transfer 50 white colonies (have vector and insert) to 50 clean eppendorf tubes containing 40µl TE Buffer

- Transfer each picked colony to a new plate with 50 marked arrays
- 3 cycles of freeze- thawing [-20°C for 20sec → 95°C 2 sec]



Forth Day

- Perform PCRs and gel electrophoresis to check fragment length
- DGGE
- Glycerin cultures

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Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

13. CURRICULUM VITAE

Name	Angelika Maria Pointner
Date of birth	18/11/1982
Place of birth	Linz, Austria

Education

Since 10/2004	Student of Nutritional Sciences at the Department of Life Sciences at University of Vienna
2001-2003	Student of Medicine at the Medical University of Vienna
06/2001	Matura with Summa cum laude – university entrance diploma

Practical experiences

Diploma thesis 2010/12	Shifts of faecal microbiota in immunosuppressed patients following chemotherapeutic treatment analyzed by TaqMan-PCR and PCR-DGGE with special focus on 454 sequencing with Univ.Doz.Dr. Alexander Haslberger
01/07-31/12/2012	Freelance co-worker at Health Bio Care GmbH
01/03/2010-31/07/2012	Tutorium University of Vienna AG Haslberger
01/06/2008-31/06/2008	Internship e3 Ernährung
Since 01/01/08	Eventmanagement Planetarium, Kuffner and Urania Sternwarte Vienna
Summer 2006	Internship Healthy Kids Camp, North Carolina, USA
01/06/05-31/12/05	Service and Kassa Planetarium Wien, Kuffner and Urania Sternwarte Vienna
Jan 04-Mai 05	Freelance co-worker SAZ-Marketing

PUBLICATIONS

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Changes in Human Fecal Microbiota Due to Chemotherapy Analyzed by TaqMan-PCR, 454 Sequencing and PCR-DGGE Fingerprinting

Jutta Zwielehner,[1] Cornelia Lassl,[1] Berit Hippe,[1] Angelika Pointner,[1] Olivier J. Switzeny,[1] Marlene Remely,[1] Elvira Kitzweger,[2] Reinhard Ruckser,[2] and Alexander G. Haslberger[1]*

[1]Department of Nutritional Sciences, Vienna, Austria

[2]Sozialmedizinisches Zentrum Ost, Vienna, Austria

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Jutta Zwielehner[1], Cornelia Lassl[1], Berit Hippe[1], Angelika Pointner[1], Olivier J. Switzeny[1], Marlene Remely[1], Elvira Kitzweger[2], Reinhard Ruckser[2], Alexander G. Haslberger[1]*

1 Department of Nutritional Sciences, Vienna, Austria, 2 Sozialmedizinisches Zentrum Ost, Vienna, Austria

Abstract

Background: We investigated whether chemotherapy with the presence or absence of antibiotics against different kinds of cancer changed the gastrointestinal microbiota.

Methodology/Principal Findings: Feces of 17 ambulant patients receiving chemotherapy with or without concomitant antibiotics were analyzed before and after the chemotherapy cycle at four time points in comparison to 17 gender-, age- and lifestyle-matched healthy controls. We targeted 16S rRNA genes of all bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV* and *XIVa* as well as *C. difficile* with TaqMan qPCR, denaturing gradient gel electrophoresis (DGGE) fingerprinting and high-throughput sequencing. After a significant drop in the abundance of microbiota ($p = 0.037$) following a single treatment the microbiota recovered within a few days. The chemotherapeutical treatment marginally affected the *Bacteroides* while the *Clostridium* cluster *IV* and *XIVa* were significantly more sensitive to chemotherapy and antibiotic treatment. DGGE fingerprinting showed decreased diversity of *Clostridium* cluster *IV* and *XIVa* in response to chemotherapy with cluster *IV* diversity being particularly affected by antibiotics. The occurrence of *C. difficile* in three out of seventeen subjects was accompanied by a decrease in the genera *Bifidobacterium*, *Lactobacillus*, *Veillonella* and *Faecalibacterium prausnitzii*. *Enterococcus faecium* increased following chemotherapy.

Conclusions/Significance: Despite high individual variations, these results suggest that the observed changes in the human gut microbiota may favor colonization with *C.difficile* and *Enterococcus faecium*. Perturbed microbiota may be a target for specific mitigation with safe pre- and probiotics.

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* E-mail: alexander.haslberger@univie.ac.at

Introduction

The human intestinal ecosystem can be pictured as a microbial organ within a host organism involving a dynamic interplay between food, host cells and microbes [1]. The microbiota plays several significant roles in the digestion of food, energy regulation, generation of short-chain fatty acids, vitamin synthesis, prevention of colonization by pathogens and protection against cell injury [2,3,4]. Moreover, the gut microbiota influences the host by directing intestinal epithelial cell proliferation and differentiation, pH, and the development of the immune system [1]. Recent culture-independent molecular studies on healthy individuals have shown that the intestinal microbiota is specific to the host [5] and resilient to modifications over time, as it is able to form an alternative stable state after disruption [6,7]. A healthy microbiota contains a balanced composition of many classes of bacteria [8]. The fecal microbiota is dominated by three groups of anaerobic bacteria: the *Clostridium coccoides* group -clostridial cluster *XIVa* (reclassified as *Blautia coccoides* [9]), the *Clostridium leptum* group - *Clostridium* cluster *IV*, and the *Bacteroides* [10,11]. 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 [12,13]. Moreover, the subgroup bifidobacteria seems to be an important part of the gastrointestinal tract (GI) microbiota, being involved in the prevention of atopic disease, obesity and insulin resistance via enhanced barrier function of the gut epithelium [14].

To prevent the invasion of endogenous bacteria from oral cavity and the GI tract into the blood stream, three defense mechanisms are considered to be relevant: innate immunity, mechanical mucosal barrier, and colonization resistance [15]. However, chemotherapy damages the rapidly generated mucosal cells of the GI and the use of antibiotics disrupts the ecological balance, allowing pathogens such as *Clostridium difficile* to grow [16,17]. This bacterium is thought to be the causative agent in up to 20% of antibiotic-associated diarrhea (AAD) cases [18]. It is evident that the intestinal microbial ecosystem has an important but incompletely defined role in mucosal protection [19].

Mucositis is a major oncological problem, caused by the cytotoxic effects of cancer chemotherapy and radiotherapy [20]. Approximately 40% of patients receiving standard dose chemotherapy and up to 100% of patients receiving high dose chemotherapy and stem cell or bone marrow transplantation suffer from abdominal pain, ulceration, bloating and vomiting [21,22]. Although gastrointestinal disturbances (mucositis, diarrhea and constipation) and immunosuppression are well recognized side-effects of cancer treatment, very little research has been conducted into the underlying mechanisms and the changes in the composition of the microbiota. Because of these changes, nutrient absorption and other intestinal functions involving the microbiota may also be altered [23].

For this reason, we investigated shifts in fecal microbiota of patients receiving cancer chemotherapy with or without antibiotics in comparison to healthy control individuals. Prescription of antibiotics may become necessary in some individuals due to bacterial infection [24]. Samples were taken at four time points before and after chemotherapy to study changes in fecal microbiota over the course of time. In this study, we aimed to clarify how chemotherapy agents influence total fecal bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV*, *Clostridium* cluster *XIVa* and *C. difficile* using culture-independent methods assessing abundance and diversity. Four samples were also analyzed with 454 high-throughput sequencing.

Results

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

DGGE fingerprinting analyses of all bacteria, *Clostridium* cluster *IV* and *Clostridium* cluster *XIVa* indicate a highly diverse dataset between individuals and uniqueness of fecal microbiota. Table 1 shows the average number of bands in cancer patients at the three time points and for controls over all time points. It becomes apparent that the average number of bands within *Clostridium* cluster *IV* declined immediately after chemotherapy (T1), followed by a recovery at T2. The average number of *Clostridium* cluster *XIVa* bands decreased after onset of chemotherapy and remained low also at T2. The datasets were subjected to principal component analysis (PCA). PCA extracts underlying components of samples according to their variance. Figure 1A illustrates the bacterial fingerprints of sample P01 over time. Figure 1B displays the PCA analysis of all bacteria. Most samples taken after chemotherapy are grouped together with all other samples. Patients who receive antibiotics are indicated as black symbols. They cluster together with the samples taken after chemotherapy and also with the majority of samples before chemotherapy and healthy controls. There are two exceptions though: Two samples from P07 after chemotherapy under antibiotic treatment are outliers in the lower right part of the PCA plot. P07 received blood stem cell transplantation resulting in a sharp decline in bacterial abundances as measured with quantitative PCR. The first two principal components explain 17.4% of variance.

Figure 1C shows the principal components analysis of *Clostridium* cluster *IV*. DGGE fingerprints of individuals after chemotherapy are found to be less variable than healthy controls and patients before onset of treatment. Although overlapping, PCA resulted in grouping of band patterns before and after chemotherapy. Additional effects by antibiotic treatment became evident: Antibiotic treatment significantly reduced the diversity within the *Clostridium* cluster *IV* ($p = 0.00003$) with Shannon diversity index being 1.4 ± 0.7 compared to patients under chemotherapy alone 2.1 ± 0.6. In the PCA plot, samples affected by antibiotics are found in the lower right corner of the plot. This means that they are grouped according to their variance along principal component (PC) 1 and 2. These two PCs explain 17.9% and 9.15% of the variance in the dataset, underlining the validity of this interpretation. Principal components analysis of *Clostridium* cluster *XIVa* is not shown.

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

To study whether chemotherapy with or without antibiotics changes the human GI microbiota composition in contrast to healthy individuals and over time, we investigated absolute numbers and relative percentages of bacterial subgroups. Absolute numbers give an indication about the direct antimicrobial effects of the treatments. Relative quantification is able to identify which bacterial subgroups are particularly affected and helps to describe the community disruption induced by chemotherapy with or without antibiotics. In absolute numbers, oncology patients harbored significantly less bacteria ($p < 0.05$) than healthy control (figure 2). From already low bacterial counts before chemotherapy, bacterial abundance significantly declined further ($p = 0.037$) immediately after chemotherapy (T1) and recovered 5 9 days later (T2) in comparison to time points before treatment (T0). Absolute numbers of bacteria in different time points of healthy controls are following a lognormal distribution in contrast to microbiota abundances in oncology patients. The decrease in total bacteria following chemotherapy ($p = 0.037$) was significantly greater than any variation in copy numbers observed in healthy controls ($p = 0.027$). The observed decrease after chemotherapy affected the *Bacteroides* ($p = 0.044$), the bifidobacteria ($p = 0.034$) and *Clostridium* cluster *IV* ($p = 0.049$) as shown in figure 3. There were also fewer absolute numbers of *Clostridium* cluster *XIVa*, but this difference was not significant. All patients with fever showed an increase in total fecal microbiota (see figure 3). In sample P07 a sharp decline affecting all bacteria and bacterial subgroups was observed at T1 (figure 3), following blood stem cell transplantation and medical intervention.

Patients who received antibiotics had highest abundances of all bacteria ($p = 0.000003$) amongst all patients (data not shown). This bacterial overgrowth affected the *Bacteroides*, the bifidobacteria and *Clostridium* clusters *IV* and *XIVa*, since relative abundances of those subgroups did not stand out significantly. Thus, patients were grouped according to their chemotherapeutic cycle regardless whether or not they received antibiotics. The influence of antibiotics on the species composition as assessed with PCR-DGGE fingerprinting is discussed in the previous section.

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

Relative quantification of *Clostridium* cluster *XIVa* as percentage of total bacterial DNA showed that oncology patients harbored significantly less *Clostridium* cluster *XIVa* ($p = 0.047$) than healthy controls. The mean proportion of *Bacteroides* in stool samples was $26 \pm 12\%$ in chemotherapy patients and $22 \pm 14\%$ in healthy individuals. The mean percentage of bifidobacteria in patients was $0.8 \pm 1.4\%$ and 0.3 ± 0.6 in controls. Patients harbored on average $16 \pm 9\%$ of *Clostridium* cluster *IV* and $18 \pm 12\%$ of *Clostridium* cluster *XIVa*, while controls harbored $20 \pm 12\%$ and $24 \pm 15\%$ of clostridial clusters *IV* and *XIVa*.

Clostridium cluster *XIVa* higher before chemotherapy than after

The mean percentage of *Clostridium* cluster *XIVa* before chemotherapy was $22 \pm 13\%$ compared to after chemotherapeutic

Table 1. Number of bands observed in PCR-DGGE fingerprinting in oncology patients before chemotherapy (T_0), immediately after chemotherapy (T_1) and 5–9 days after chemotherapy (T_2) and healthy controls averaged over all time points.

Time point	All bacteria	*Clostridium* cluster *IV*	*Clostridium* cluster *XIVa*
T_0	18.9±4.6	14±7.0	8±3.2
T_1	19.7±4.9	10±6.0	4.9±3.6
T_2	19.6±3.6	15±6.0	5.2±2.6
control	19.2±3.5	12.0±5.0	8.9±3.0

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cycles with 19±12%. The average amount of *Bacteroides*, bifidobacteria and *Clostridium* cluster *IV* were 26±11%, 1.4±2% and 16±9% at time points before chemotherapy and 28±14%, 0.5±1.2% and 18±12% after chemotherapy. Figure 3 illustrates the development of the microbiota in the course of antibiotic treatment. Data were normalized for clarity, so that changes in abundances from time point T0 (before onset of treatment) to T1 (1 4 days after chemotherapy) and T2 (5 9 days after chemotherapy) rather than relative abundances are shown. It can be seen that chemotherapy causes a dramatic reduction of microbiota abundance immediately after chemotherapy, affecting all subgroups. As mentioned above, the significant decrease in all bacteria following chemotherapy was significantly greater than any variation in copy numbers observed in healthy controls ($p = 0.027$).

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

To find out whether the chemotherapeutic and antibiotic disruption favors the growth of pathogens, we investigated the abundance of *C. difficile*. Three out of seventeen patients receiving chemotherapy harbored *C. difficile* (data not shown). Patient P09 harbored C. difficile at all time points investigated. Mean proportion over all four samples of P09 was recorded as 0.4±0.7%, yet the highest level (1.22% of total bacteria) occurred at sampling point T1 immediately after chemotherapeutic and antibiotic treatment. *C. difficile* was detected in P11 (3.90% of all analyzed bacteria) after chemotherapeutic intervention at time point T1. P14 carried *C. difficile* in low abundance directly after onset of chemotherapy (0.003% of all analyzed bacteria). Samples of patients P09 and P11 at T0 and T1 were further analyzed in 454 sequencing.

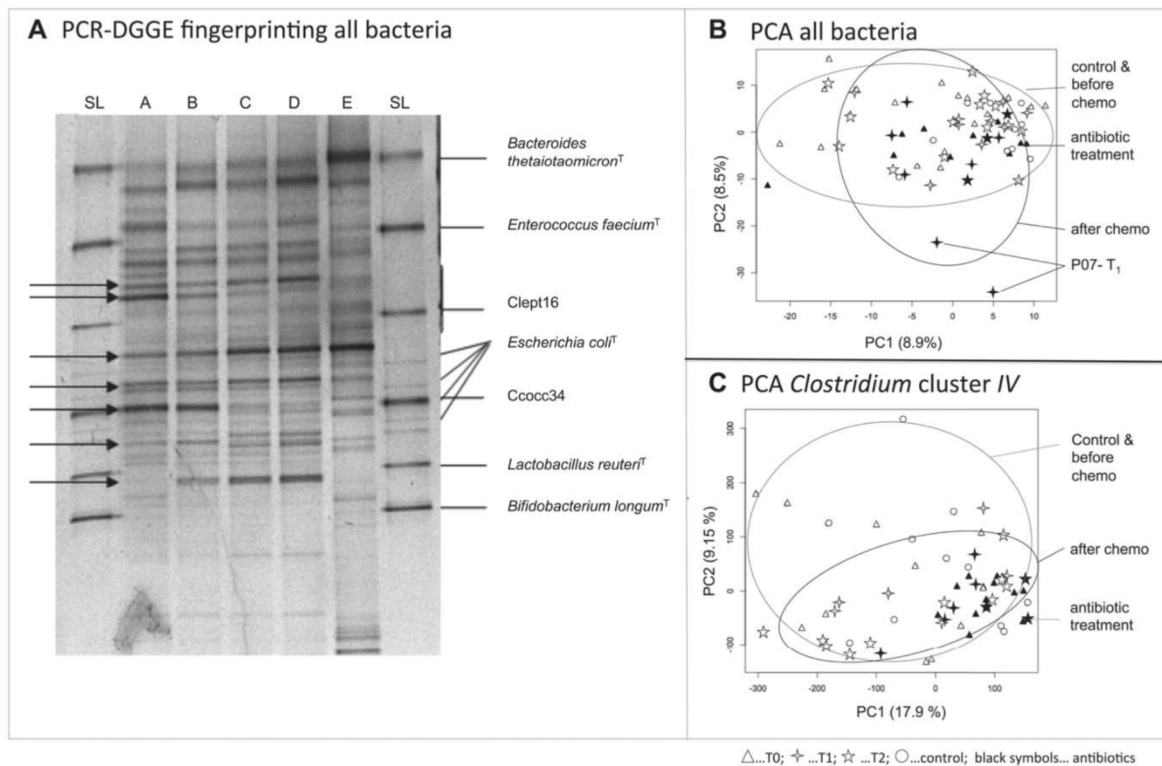

Figure 1. A PCR-DGGE fingerprinting of 16S rRNA coding regions of dominant bacteria over time. Bands that become stronger or nearly disappear following a single chemotherapeutic treatment are indicated with arrows. B Principal components analysis (PCA) based on dominant bacteria PCR-DGGE fingerprinting. The two outliers in the lower right corner of the plot are two samples of P07 following blood stem cell transplantation. C PCA illustrating the development of *Clostridium* cluster *IV* diversity in the course of chemotherapy and antibiotic treatment. Cluster *IV* diversity drops right after chemotherapy, causing a grouping of samples. Samples under antibiotic treatment (indicated as grey dots) group even closer, indicating a strong influence of antibiotics on *Clostridium* cluster *IV* diversity. A, sample of P01 before chemotherapy B, C and D, samples of P01 after chemotherapy; E, healthy control; SL, unrelated standard lane; black symbols… patients under chemotherapy and antibiotic treatment.
doi:10.1371/journal.pone.0028654.g001

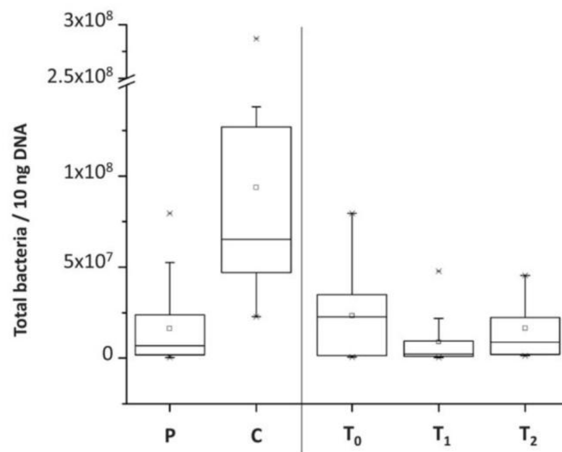

Figure 2. TaqMan qPCR quantification of bacterial 16S rRNA coding regions showing lower abundance in patients undergoing chemotherapy and antibiotic treatment (P) than healthy controls (C). T_0, samples taken before a single shot of chemotherapy; T_1, 1–2 days after chemotherapy; T_2, 5–9 days after chemotherapy; Asterisk indicates a significant difference at $p<0.05$.
doi:10.1371/journal.pone.0028654.g002

High throughput sequencing

High throughput sequencing showed a dramatic increase in sequences within the *Peptostreptococcaceae* towards sequences 98.9 100% similar to *C.difficile*^{T} (figure 4): *Clostridium bartletti* related sequences (98.1 100% similarity) were only detected before chemotherapy (T0). After chemotherapy (T1), 63 sequences 98.9 100% similar to *C.difficile* appeared in samples P11 and P09. In accordance with the phylogenetic classification by the ribosomal database project, they are shown as 'unclassified *Peptostreptococcaceae*' in figure 5.

Furthermore pronounced reductions of *Faecalibacterium* spp. as well as lactobacilli, *Veillonella* spp., bifidobacteria (in P09) and *E.coli/Shigella* became apparent in response to chemotherapy (figure 5). The abundance of lactobacilli decreased in both patients after chemotherapy, in P09 from already low levels. Individual P11 did not receive concomitant antibiotics, whereas P09 did. In P09 and P11 *Faecalibacterium* spp. decreased dramatically from 9.5% and 8.3% to 0.07% and 0.00%, respectively. In both individuals *Enterococcus faecium* increased following chemotherapy. Furthermore, less abundant sequences appeared that were attributable to bacterial genera not detected before chemotherapy. These genera are: *Eggerthella*, *Megasphaera*, *Parvimonas* (only in P11), *Anaerostipes*, *Eubacterium*, *Anaerococcus*, *Methylobacterium*, *Holdemania*, *Turicibacter*, *Akkermansia*, *Sutterella* (only in P09), *Sphingomonas*, *Anaerotruncus*, *Coprococcus*, *Streptococcus* and *Dorea*. Species with abundance <0.01% of all sequences are not shown in figure 5. The number of *Blautia* species from *Clostridium* cluster *XIVa* remained constant in the 454 sequencing datasets before and after chemotherapy.

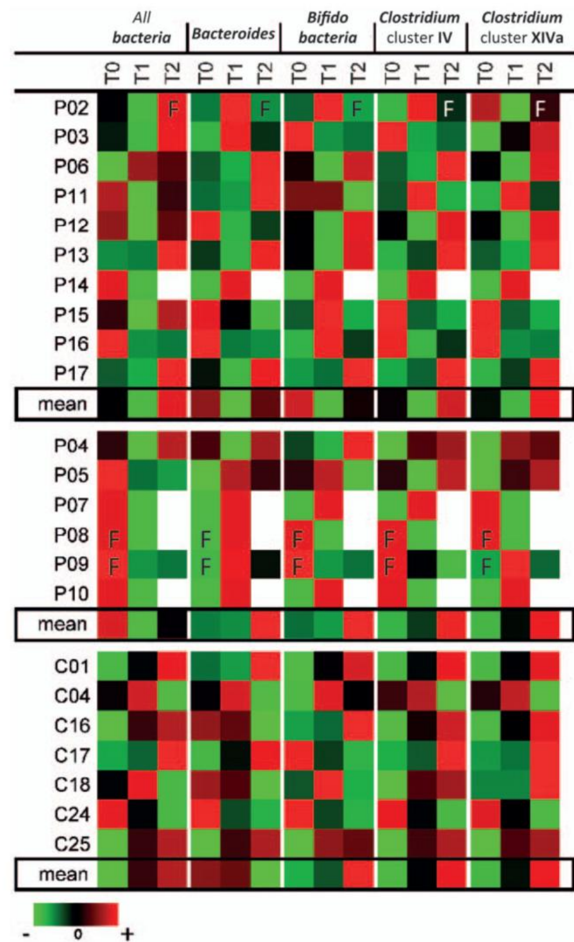

Figure 3. Abundances of bacterial 16S rRNA coding regions over time in oncology patients (P) and healthy controls (C). The declined abundances of bacteria, *Bacteroides*, *Clostridium* cluster *XIVa*, *Clostridium* cluster *IV* and bifidobacteria immediately after chemotherapy (T_1) were observed to recover several days after treatment (T_2). Patients P04, P08 and P13 had never received chemotherapy before; P04, P05, P07, P08, P09 and P10 took antibiotics. Values were z-scored for presentation in this heatmap showing changes over time rather than absolute abundances. T_0, before chemotherapy; T_1, 1–2 days after chemotherapy; T_2, 5–9 days after chemotherapy; F, fever; S, blood stem cell transplantation.
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Discussion

Chemotherapeutic and antibiotic use is associated with severe side effects such as mucositis, diarrhea and constipation. These side effects increase the cost of health services and are often life threatening [22]. Chemotherapeutic and antibiotic treatment has a detrimental impact on the host microbial ecosystem, which is important for host mucosal protection [19] and thereby increases the risk of infection [17]. Overgrowth of species with potential pathogenicity such as toxigenic *C. difficile* and inflammatory complications are among the most common serious complications of chemotherapy and antibiotic treatment among patients with cancer [15,17].

We investigated how the use of cancer chemotherapy (in some individuals together with antibiotic treatment) perturbs the fecal microbial ecosystem during the course of therapy. We assessed if the microbiota is able to return to its original profile after chemotherapeutic and antibiotic intervention with special interest in the abundance of *C. difficile*. We used a combination of molecular methods including high-throughput sequencing to compare diversity (PCR-DGGE) and abundance (qPCR) of all

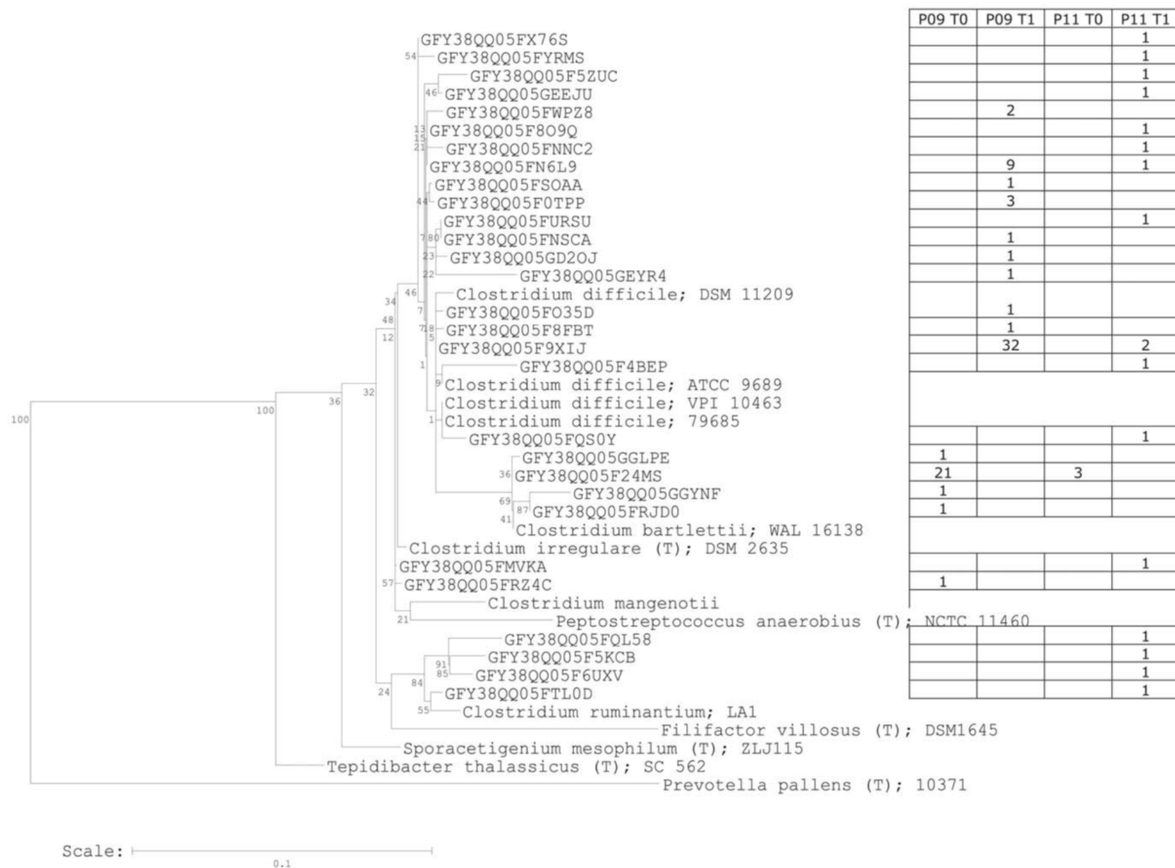

Figure 4. Phylogenetic tree showing the *Peptostreptococcaceae* found in samples from two oncology patients before and after chemotherapy. Identical sequences were grouped; the table on the right hand side shows their abundances in the 454 sequencing dataset. Sequences with >98.9% similarity to *Clostridium difficile* appeared only in samples taken immediately after chemotherapeutic cycles. Numbers indicate bootstrap values after 100 resamplings.
doi:10.1371/journal.pone.0028654.g004

bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV*, *Clostridium* cluster *XIVa* and *C. difficile* between groups and different time points of chemotherapy. The majority of previous studies on the effect of chemotherapy on human fecal microbiota used standard microbiological culture techniques [16,22]. Other studies have focused on the colonization of pathogenic bacteria [17,25] in patients with cancer and chemotherapy-induced diarrhea [22,26]. As mentioned above, we used feces as source of information. Fecal microbial communities are composed of autochthonous gut members and transient bacteria. Even though the fecal microbiota might be different from the adherent microbiota, we chose fecal samples to investigate the microbial composition of the intestinal microbiota because they are easy to collect, are less invasive and reflect shifts in microbial population composition [11].

In this study, we assessed species richness using PCR-DGGE fingerprinting. Each lane of a PCR-DGGE gel represented a microbial fingerprint of a fecal sample; each band within a lane corresponded to one bacterial species, although different species may sometimes be represented by the same band [17]. It has also been observed that one bacterial strain may form several bands due to multiple 16S rRNA operons, e.g. *E.coli* (figure 4A). The limitations of DGGE in microbial analysis have been previously described [27]. Nevertheless, substantial information about species composition can be obtained from very complex microbial communities such as the gut microbiota [27]. We found decreased species richness immediately after the chemotherapeutic shot, especially within *Clostridium* cluster *IV* where the number of different bands decreased from 14 ± 7 before chemotherapy (T_0) to 10 ± 6 bands shortly after (T_1). The microbiota recovered to a richness of 15 ± 6 *Clostridium* cluster *IV* bands per individual, but at a different composition, as evidenced by the grouping of samples in principal components analysis.

For quantification of fecal microbiota we used the strains *Bacteroides thetaiotaomicron*^{T}, *Bifidobacterium longum* ssp. *longum*^{T} and *C. difficile* as well as the clones CL16 and CC34 as standards. However, a mixture of different strains for qPCR standards might result in a more accurate image of the human microbiota. Therefore absolute amounts should be considered as semi-quantitative.

Grouping oncology patients with and without antibiotic treatment poses a risk to falsely interpret the effects of antibiotic treatment as effects of chemotherapy. Patients who received antibiotics had significantly higher bacterial abundances than patients without antibiotics. This observation might be the reason

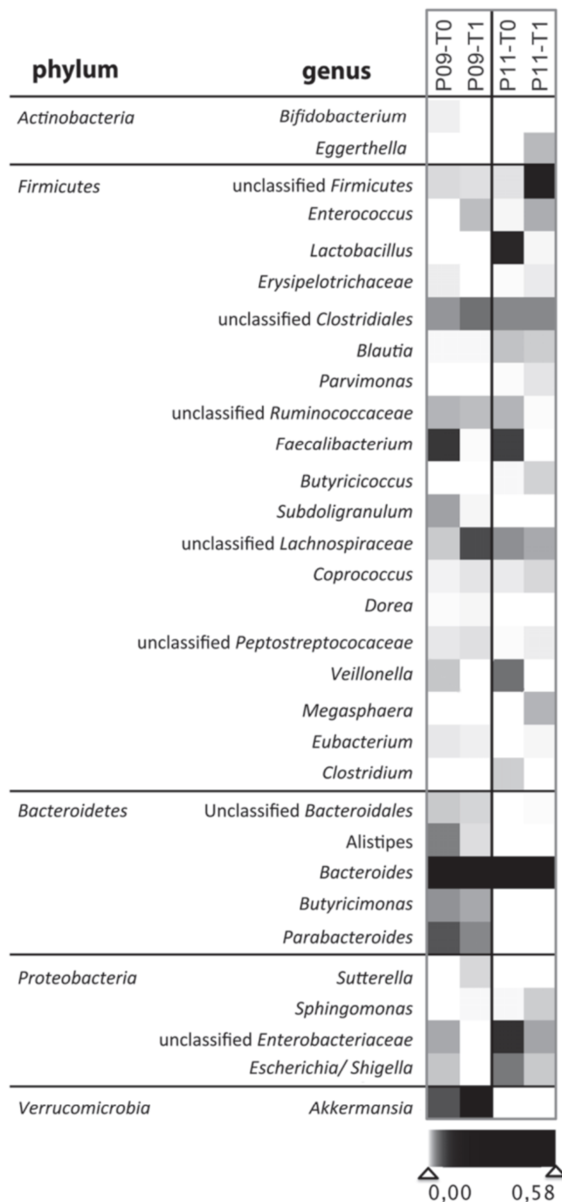

Figure 5. Heatmap showing abundances within the 454 sequencing dataset on the genus level. High throughput sequencing of samples P09 and P11 before (T_0) and after therapy (T_1) further helped to characterize the influence of a single chemotherapeutic cycle on the GI-microbiota. P11 was treated with chemotherapy alone and P09 also received antibiotic treatment.
doi:10.1371/journal.pone.0028654.g005

for antibiotic treatment rather than its effect [24]. The abundance of bacterial subgroups, also *Clostridium* cluster *IV*, changed together with total bacteria both in patients with and without antibiotics. The sharp reduction of bacteria immediately after chemotherapy equally affected patients with and without antibiotics. In PCR-DGGE analysis we found that the all bacteria and *Clostridium* cluster *XIVa* fingerprints did not differ significantly in patients *with* or *without* antibiotics. This indicates that the use of antibiotics does not fully explain the observed changes. Previous work [17] has also found additional effects of chemotherapy in cases under prophylactic antibiotic treatment. Although the *Clostridium* cluster *IV* abundance did not differ significantly due to antibiotics, PCR-DGGE fingerprints showed grouping of patients under antibiotic treatment in principal components analysis.

Despite high individual variations, we show a significantly lower absolute bacterial load in feces of patients receiving chemotherapy in comparison to healthy controls. These findings are in line with data from van Vliet *et al.* (2009) who reported 100-fold lower total bacterial numbers during chemotherapy than in healthy controls.

The abundance of fecal microbiota decreased after a single cycle of chemotherapy. After the end of chemotherapeutic administration the bacterial abundance recovered within a few days, sometimes even showing a "rebound-effect" with numbers elevating above initial levels. Relative numbers of *Clostridium* cluster *IV* and *XIVa* showed great alterations due to chemotherapeutical interventions, while the bifidobacteria seemed to be less affected. In agreement with previous results [16] increased counts of *Bacteroides* spp. were found in patients undergoing chemotherapy. Nyhlèn *et al.* (2007) also reported significant increases in yeast in patients, making it a focus for further research in immunocompromised patients. Samples taken immediately after chemotherapy had a lower diversity within *Clostridium* cluster *IV*. Antibiotics strongly contributed to the reduced diversity of cluster *IV* but were not alone responsible for this effect. A few days later we observed a quantitative recovery, but not a recovery of the composition as evidenced by clustering of DGGE fingerprints.

The incidence of *C. difficile* in subjects P09 and P11 immediately after chemotherapy is accompanied by a decrease of the genera *Bifidobacterium*, *Lactobacillus* and *Clostridium* cluster *IV*. Sequences attributable to *Faecalibacterium prausnitzii* decreased dramatically from 9% to zero. The anti-inflammatory *F. prausnitzii* was associated with dietary fiber in colonic fermentation of healthy subjects [28] and found at low abundance in individuals suffering from inflammatory bowel diseases [29] [30,31]. *Enterococcus faecium* increased following chemotherapy, possibly filling the ecological niches vacated by the lactobacilli and bifidobacteria. *Enterococcus faecium* is a facultative pathogenic bacterium causing life-threatening infections especially in nosocomial settings [32]. *Enterococcus faecium* has previously been found to increase in wastewater upon treatment [33]. The acquisition of multi-resistant *E. faecium* strains has been described in hospital environments under high selective antibiotic pressure. Under such conditions probiotic strains were demonstrated as unable to prevent nosocomial infection [34].

After chemotherapy less abundant sequences appeared that were not detected before treatment. These genera are: *Eggerthella*, *Megasphaera*, *Parvimonas*, *Anaerostipes*, *Eubacterium*, *Anaerococcus*, *Methylobacterium*, *Holdemania*, *Turicibacter*, *Akkermansia*, *Sutterella*, *Sphingomonas*, *Anaerotruncus*, *Coprococcus*, *Streptococcus* and *Dorea*. *Eggerthella lenta* was described to convert dietary lignans to the bioactive enterolactone [35]. *Megasphaera* spp. have been described as propionate-producers that utilize lactate [36] comparable to *Veillonella* spp. that were no longer detected by 454 sequencing after chemotherapy. The butyrate-producing *Anaerostipes caccae* and *Eubacterium hallii* utilize lactate as well. They were suggested to compete for lactate with sulfate-reducing bacteria such as *Desulfobacter piger* whose preferred co-substrate is lactate. High concentrations of sulfate are toxic for the gut epithelium and may contribute to bowel disease [37]. Microorganisms of the genus *Methylobacterium* are facultative methylotrophic, gram-negative rods that are ubiquitous in nature and rarely cause human disease, except in subjects with pre-existing immunosuppression. For

instance, in 2010, a case of *M. fujisawaense* infection was described in a patient with relapsed acute leukemia undergoing unrelated allogeneic hematopoietic stem cell transplantation [38]. *Turicibacter* is a poorly known genus previously found in weaned piglets, known to be susceptible to chlortetracycline [39]. In humans, *Turicibacter* spp. have been found in the ileal pelvic pouch of a former ulcerative colitis patient [40]. *Akkermansia muciniphila* is a common mucin-degrading bacterium of the human GI. Its prevalence has been described to be 10^8 cells/g feces in adults, decreasing with age [41]. *Dorea* spp. are mucosa-associated bacteria of the human GI that are members of the *Clostridium coccoides* rRNA group of organisms [42,43].

Further research is needed to elucidate if there is a causal relationship between growth of *C. difficile* and decreased abundance of lactobacilli, bifidobacteria and *Clostridium* cluster *IV*, especially the anti-inflammatory *Faecalibacterium prausnitzii*. The increase of mucus-degrading bacteria might be a result of *C.difficile* and probably also *E. faecium* associated inflammation of the gut epithelium. Mucus hypersecretion is a common symptom of irritable bowel syndrome, ulcerative colitis and bacterial infections of the gut epithelium [44,45]. The lactate-utilizing microbiota shifted from *Veillonella* spp. to *Anaerostipes*, *Eubacterium* and *Megasphaera* spp. This change may be interpreted as a beneficial adaptation, because lactate could otherwise be used as a co-substrate for sulfate-reduction. Sulfate-reducing bacteria, however, were not detected here.

The oncology patients assessed here suffered from a variety of malignancies and received different chemotherapy treatment regimes. Only two participants (P01 and P08) had never received any cancer therapy before, while all others had a history of chemotherapeutic treatment. Therefore, the observed changes are likely to be influenced by previous cycles of chemotherapy. For example, the significantly lower bacterial abundance in cancer patients before chemotherapy in comparison to control could be a consequence of previous treatments. The results presented here illustrate changes due to a single chemotherapeutic cycle, but cannot rule out, that these changes occurred as a consequence of several chemotherapeutic cycles over the course of several years. Six cancer patients also received antibiotics. These patients were characterized by significantly elevated abundances of bacteria. This finding confirms the diagnosis 'bacterial infection' for which antibiotic treatment was prescribed. *Clostridium* cluster *IV* PCR-DGGE profiles revealed a shift in species composition by chemotherapy, and even more so by antibiotics. Thus we conclude that antimicrobial treatment significantly reduces the species richness of the *Clostridium* cluster IV, with the anti-inflammatory *Faecalibacterium prausnitzii* being the most abundant representative. Van Vliet *et al.* (2009) tested the effect of chemotherapy *in vitro* and showed a direct bacteriostatic effect of chemotherapeutics on bacterial growth.

Further research is needed to show whether changes in bacterial colonization play a role in the development and maintenance of mucosal barrier function, infection and inflammation [17].

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

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

Materials and Methods

Ethics statement

The Viennese Human Ethics committee (3., Thomas-Klestil-Platz 8/2) under the chair of Dr. Karin Spacek, approved the proposal of the project "Analysis of microbiota in feces of patients with immunosuppression". Votum: EK 07-153-VK, 2008. From all participants involved in the study written consent was obtained.

Study participants and study design

Seventeen subjects receiving ambulant chemotherapy with or without antimicrobial therapy (aged 59±13 y, BMI 27±6) from the Sozialmedizinisches Zentrum Ost (SMZ Ost) in Vienna and seventeen healthy individuals (aged 65±18 y, BMI 24±5) joined this study. Four fecal samples within two weeks were collected of each ambulant oncology patient in order to collect samples before and after a single immune-suppressive chemotherapy cycle. The four samples obtained from every patient were grouped into three groups: samples taken before the day of chemotherapy (T0), samples taken 1 4 days after chemotherapy (T1) and samples taken 5 9 days after chemotherapy (T2). Healthy individuals also donated four samples during two weeks. Gender ratio among healthy controls was 56% female, 44% male. Oncology patients were 47% female and 53% male. Three out of seventeen patients (P04, P08, P13) had never received any chemotherapy before, while the others had a history of chemotherapy. Anonymous medical records reported types of malignancies as well as chemotherapeutic and antimicrobial treatment as shown in table 2.

We interviewed all study participants assessing age, gender, body length, weight, health status (chronic and acute diseases), and life-style aspects such as alcohol consumption and physical activity. Dietary habits were assessed using a food frequency questionnaire. Exclusion criteria for healthy controls were (a) antimicrobial medication (b) chemotherapeutic treatment and (c) pre- and probiotics at least three months before sample collection. Approval for this study was obtained from the Viennese Human Ethics committee (3., Thomas-Klestil-Platz 8/2).

Stool sample processing

After collection, study participants immediately stored their samples at -18°C in their homes. Stool samples were still frozen when brought to the laboratory and then immediately stored at −70°C. A 200 mg aliquot of each sample was treated twice for 45 s in a bead-beater (Mini-Beadbeater-8). Thereafter DNA was extracted using the QIAamp® DNA Stool Mini Kit (QIAGEN) following the manufacturer's protocol. The DNA was stored at −20°C until analysis.

Type strains

We used type strains, known to be part of the human gastrointestinal microbiota and cloned sequences to design a DGGE standard lane marker. Type strains *Bacteroides thetaiotaomicron* DSM 2079T, *Enterococcus faecium* DSM 20477T, *Lactobacillus reuteri* ATCC 55730T, *Bifidobacterium longum* ssp. *longum* DSM 20097T, *Escherichia coli* IMBH 252/07 and clones CL16 and CC34 (see below) were used for creating a comparable standard lane marker for DGGE gels analyzing all bacteria. *E.coli* IMBH 242/07 gave 4 bands due to its multiple operons for the 16S rRNA gene.

Clone library

To create a standard lane marker for DGGE analysis and to identify members of the *Clostridium* cluster *XIVa* we constructed clone libraries from two stool samples of healthy volunteers. For this purpose PCR products amplified with primers 195-F [46] and

Table 2. Relevant clinical data of study participants undergoing immunesuppressive chemotherapy.

name	diagnosis	chemotherapeutic treatment	antimicrobial treatment	other condition
P01	urothel carcinoma	gemcitabine, cisplatinum		
P02	plasmocytoma, multiple myeloma	bortezomib, dexamethasone		rheumatism fever at 4th sampling
P03	Non-Hodgkin lymphoma	bendamustine		diabetes II, adipositas, hypertension
P04	ovarian fibroma	taxol, carboplatin	levofloxacin	
P05	multiple myeloma	bortezomib, doxorubicin, dexamethasone	cotrimoxazole	osteoporosis
P06	mamma carcinoma	pegylated liposomal doxorubicin hydrochloride, gemcitabine		
P07	Non-Hodgkin lymphoma	high dose radiation therapy and PBSCT	cotrimoxazole, piperacillin, tazobactam	fever at 2nd and 3rd sampling
P08	monozytic leukemia	cytarabine, idarubicin	cotrimoxazole, piperacillin, tazobactam	fever at 2nd sampling
P09	acute leukemia	high dose Ara-C, radiated erythrocyte concentrate	piperacillin, tazobactam	fever at 2nd sampling
P10	Non-Hodgkin lymphoma	ifosamid, etoposid, methotrexat	levofloxacin	
P11	Acute lymophoblastic leukemia	cytarabine, methotrexat		adipositas, hypertension
P12	small intestinal tumor	cetuximab		
P13	rectal tumor	capecitabine, oxaliplatin		
P14	thymus tumor	taxol, carboplatin, bevacizumab, radiation		
P15	Acute lymophoblastic leukemia	cyclophosphamide, methotrexate, doxorubicin, cytarabine, vincristine		diabetes II
P16	Acute lymophoblastic leukemia	cytarabine, mitoxantrone		
P17	colon tumor	oxaliplatin, capecitabine, bevacizumab, irinotecan, monoclonal antibodies		

PBSCT… peripheral blood stem cell transplant.
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Ccocc-R [47] were inserted into a p-GEM Easy Vector (Promega) following the instructions of the manufacturer. Nucleotide sequences were corrected for primer and vector sequences in CodonCodeAligner (www.codoncode.com) and taxonomically identified using the online tools of the ribosomal database project (http://rdp.cme.msu.edu/). The clone library used for creating a standard lane marker for DGGE analysis of *Clostridium* cluster *IV* has previously been described [48]. Clones CL16 (*Clostridium leptum* 16) and CC34 (*Clostridium coccoides* 34) were also used as positive controls in Taqman qPCR.

Polymerase chain reaction (PCR)

PCR was carried out amplifying 16S rRNA gene sequences from bacteria in fecal samples, type strains and cloned sequences for DGGE analysis as well as for creation of the clone library using group- and kingdom- specific primers (table 3). The PCR reaction mixture consisted of ready-to-use mastermix (Promega) with 1.5 mM $MgCl_2$, 500 nM of primers and 2 µl of template DNA. When amplifying fecal samples, bovine serum albumin (Fermentas) was added to a final concentration of 400 µg/ml. We used a Robocycler (Stratagene) for all amplifications.

PCR-DGGE-fingerprinting

DGGE was performed as previously described [49]. Primer pairs and annealing temperatures to analyze the diversity of (a) all bacteria, (b) *Clostridium* cluster *IV* and (c) *Clostridium* cluster *XIVa* are described in table 3. 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*. Electrophoresis was performed for 9 h at 129 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*). Standard lane markers were created for each DGGE analysis assay to ensure reliable gel-to-gel comparison. These standard lane markers (described above) were loaded in triplicate on each gel to adjust for gradient-variations between gels. We analyzed PCR-DGGE fingerprints using GelComparII (www.applied-maths.com). When generating the band comparison, a 1% tolerance was selected. Principal components analysis (PCA) was applied on quantitative band comparison datasets in 'R' (www.r-project.org) using the default settings. Shannon diversity index was calculated on quantitative band information as well with the default settings implemented in the 'vegan' package in 'R' (www.r-project.org). Shannon index is defined as $H = -\sum pi \ln pi$, where pi is the proportional abundance of species i. In short, the higher the Shannon index is, the higher is the diversity. For interpretation of results, samples were grouped into three groups: samples taken before the day of chemotherapy (T0), samples taken 1 4 days after chemotherapy (T1) and samples taken 5 9 days after chemotherapy (T2).

Quantitative TaqMan qPCR

The abundance of bacteria and bacterial subgroups was measured by 16S rRNA gene-targeting TaqMan qPCR in a Rotorgene 3000 (Corbett Life Science). Primers, annealing temperatures and expected product sizes are shown in table 4. Each sample was analyzed in duplicate. Amplifications were carried out in a total volume of 10 µl consisting of 5 µl Taq-Man SensiMix DNA Kit (Quantance), 1 µl of each primer and Taq-Man probe (concentrations see table 4) and 10 ng of bacterial

Table 3. 16S rRNA gene primers used for PCR-DGGE fingerprinting.

Target organism	Primer	Sequence (5′-3′)	Ann. temp (°C)	Reference
All bacteria	27f	GTGCTGCAGAGAGTTTGATCCTGGCTCAG	57	[52 T., Blocker 1989]
	985r	GTAAGGTTCTTCGCGTT	57	[53]
	341f-GC	CCT ACG GGA GGC AGC AG	55	[49]
	518r	ATT ACC GCG GCT GCT GG	55	[54]
Clostridium cluster IV	sg-Clept-F-GC	GCA CAA GCA GTG GAG T	55	
	sg-Clept-R3	CTT CCT CCG TTT TGT CAA		[55]
Clostridium cluster *XIVa*	Ccocc-F-GC	AAATGACGGTACCTGACTAA	55	
	Ccocc-R	CTTTGAGTTTCATTCTTGCGAA		[55]
GC-clamp		CGCCCGGGGCGCGCCCCGGGCGGCCCGGGGGCACCGGGGG		[49]

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DNA. Amplification programs included an initial denaturation at 95°C for 10 min followed by 40 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C (all bacteria, *Clostridium* cluster *IV*), 56°C (*Clostridium* cluster *XIVa*), 58°C (*C. difficile*) or 60°C (*Bacteroides*, bifidobacteria) for 30 s and extension at 72°C for 50 s.

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

We quantified DNA of *Bacteroides thetaiotaomicron*^{T}, *Bifidobacterium longum* ssp. *longum*^{T} and C. difficile, using the nanodrop method and calculated DNA copies/µl through mean G+C content of each strain. Clones CL16 and CC34 were amplified with the SP6 Promoter Primer (Promega, Cat.# Q5011) and the T7 Promoter Primer (Promega, Cat.# Q5021) and the PCR product quantified using a nanodrop machine. Knowing the sequences of these two PCR products and their flanking vector sequences we could quantify the copy numbers and use it as standards. 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 [50].

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

High throughput sequencing

In total, four samples (P09-T0, P09-T1, P11-T0, P11-T1) were amplified with primer 525F (5′- TCAGCAGCCGCGGTAATAC -3′) and 926R (5′-TCCGTCAATTCCTTTGAGTTT -3′) using a high-fidelity DNA polymerase (Phusion®, Finnzymes, Thermo Fisher Scientific) and submitted to 454 barcode sequencing (AGOWA, Berlin, Germany), resulting in a total of 113 000 reads. The sequences were trimmed and aligned using the pyro

Table 4. Primers and probes used for TaqMan qPCR quantification of 16S rRNA genes.

Target organism	Primer and probe	Sequence (5′ - 3′)	Size (bp)	Conc. (nM)	Reference
Bifidobacterium spp.	Forward primer	GCG TGC TTA ACA CAT GCA AGT C	125	300	
	Reverse primer	CAC CCG TTT CCA GGA GCT ATT		300	
	Probe	(FAM)- TCA CGC ATT ACT CAC CCG TTC GCC -(BHQ-1)		150	[56]
Bacteroides	AllBac296f	GAG AGG AAG GTC CCC CAC	106	300	
	AllBac412r	CGC TAC TTG GCT GGT TCA G		300	
	AllBac375Bhqr	(FAM)-CCA TTG ACC AAT ATT CCT CAC TGC TGC CT-(BHQ-1)		100	[53]
All bacteria	BAC-338-F	ACT CCT ACG GGA GGC AG	468	1000	
	BAC-805-R	GAC TAC CAG GGT ATC TAA TCC		1000	
	BAC-516-P	(FAM)-TGC CAG CAG CCG CGG TAA TAC-(BHQ-1)		200	[50]
Clostridium cluster *IV*	sg-Clept-F	GCA CAA GCA GTG GAG T	239	400	
	sg-Clept-R3	CTT CCT CCG TTT TGT CAA		400	[47]
	Clept-P^{++}	(FAM)-AGG GTT GCG CTC GTT-(BHQ-1)		200	This study
Clostridium cluster *XIVa*	195F	GCA GTG GGG AAT ATT GCA		500	[46]
	CcoccR	CTT TGA GTT TCA TTC TTG CGA A		500	
	CcoccP	(6-FAM)-AAATGACGGTACCTGACTAA-(BHQ-1)		150	[47]
Clostridium diffficile	CdiffF	TTG AGC GAT TTA CTT CGG TAA AGA		1000	[56]
	CdiffR	TGT ACT GGC TCA CCT TTG ATA TTC A	151	1000	
	CdiffP	(6-FAM)-CCA CGC GTT ACT CAC CCG TCC G-(BHQ-1)		200	

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pipeline of the ribosomal database project (http://rdp.cme.msu.edu/). Only sequences longer than 150 bp were retained, resulting in 3886 to 6811 sequences per sample with the average lengths of 366 to 368 bp. All analyses were performed using the online tools of the ribosomal database project pyro pipeline (http://rdp.cme.msu.edu/). Results of the phylogenetic classification are shown as a heatmap [51]. The *Peptostreptococcaceae*, harbouring also *C.difficile*, from all four datasets were analyzed in more detail using the online tools of the ribosomal database project. 100% similar sequences were grouped and their abundances shown together with a phylogenetic tree.

Data analysis

Statistical evaluation of differences between groups (chemotherapy and control) and changes within the chemotherapy group (all time points before and after chemotherapy) was carried out using the OriginPro version 8 (OriginLab, Northampton, MA). For two group comparisons of independent ordinal and interval values we used the two-sample t-test and the nonparametric Mann-Whitney U-test. For the analysis of related data we used the paired sample t-test or the non-parametric Wilcoxon signed-rank test. P values <0.05 were considered statistically significant. To show the decline in abundance immediately after chemotherapy qPCR results were plotted in heatmaps [51]. Values were z-scored for presentation in this heatmap showing changes over time rather than absolute abundances.

Dietary aspects

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

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Author Contributions

Conceived and designed the experiments: JZ RR AGH. Performed the experiments: JZ AP CL BH OJS. Analyzed the data: JZ CL BH AP OJS RR AGH. Contributed reagents/materials/analysis tools: EK MR RR. Wrote the paper: JZ AGH.

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