Dissertation

Biogeography of the human gastro-intestinal microbiota in health and disease and its reconstitution by fecal microbiota transplantation

submitted by

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under the Supervision of

Prof. Dr. Gregor GORKIEWICZ

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

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Abbreviations and Definitions

AAA enterocolitis	Antibiotic-associated apoptotic enterocolitis
AB	Antibiotic
AD	Alzheimer's disease
AMP	Antimicrobial peptide
ANOSIM	Analysis of similarities
ARDS	Acute respiratory distress syndrome
ASD	Autism spectrum disorder
bp	base pair
BSS	Bristol stool scale
CD	Crohn's disease
CDI	Clostridium difficile infection
CNS	Central nervous system
CoA	Coenzyme A
CRC	Colorectal cancer
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulfate
EHEC	Enterohemorrhagic *E. coli*
EMP	Earth Microbiome Project
FDR	False discovery rate
FMT	Fecal microbiota transplantation
GC-MS	Gas chromatography - mass spectrometry
GERD	Gastroesophageal reflux disease
GI	Gastro-intestinal
GPR43	G protein-coupled receptor 43
GvHD	Graft versus host disease
HCl	Hydrochloric acid
HGP	Human Genome Project
HMP	Human Microbiome Project
HP	Helicobacter pylori
HVR	Hypervariable region
IBD	Inflammatory bowel disease
IHC	Immunohistochemistry
ICU	Intensive care unit
ITT	Intention to treat
kb	kilo base pairs
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size

MCP-1	Monocyte chemotactic protein-1
MID	multiplex identifier
MOF	Multiple organ failure
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NR	No response
nt	nucleotide
OMV	Outer membrane vesicle
OTU	Operational taxonomic unit
PCoA	Principle Coordinates Analysis
PCR	Polymerase chain reaction
PD	Parkinson's disease
PP	Per protocol
PPI	Proton pump inhibitor
PR	Partial response
PSA	Polysaccharide A
qid	Four times a day
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
RDP	Ribosomal Database Project
RE	Remission
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCFA	Short-chain fatty acid
SEM	Standard error of the mean
SOP	Standard Operating Procedure
T1D	Type 1 diabetes
T2D	Type 2 diabetes
Th	T helper cells
tid	Three times a day
TNF	Tumor necrosis factor
Tregs	Regulatory T-cells

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Zusammenfassung

Zahlreiche Studien haben gezeigt, dass klinische Eingriffe im Magen zu Veränderungen der fäkalen Mikrobiota führen, was auf eine wichtige Rolle des Magens bei der gastrointestinalen (GI) Homöostase hinweist. Diese Arbeit charakterisiert die Magen-Mikrobiota inkl. Schleimhaut von Ösophagus und Duodenum mittels 16S rRNA Amplikon-Sequenzierung und vergleicht sie der Magen-Mikrobiota von Mäusen und Wüstenrennmäusen. Die Mikrobiota des Magenaspirats beim Menschen wird von Firmicutes und Bacteroidetes dominiert. Beide Fraktionen weisen große Unterschiede auf, die in keinem Zusammenhang mit dem Gesundheitszustand der Patienten stehen. Die Zusammensetzung der Magen-Mikrobiota ist vom mikrobiellen Einstrom aus dem Ösophagus abhängig. In der Magenschleimhaut werden spezifische Bakterien sequentiell angereichert (*Rothia mucilaginosa*, *Porphyromonas* und *Lachnospiraceae*) oder abgebaut (*Streptococcus*). Die taxonomische Zusammensetzung der Magen-Mikrobiota weist große Unterschiede in Wüstenrennmäusen und Mäusen im Vergleich zum Menschen auf. Während alle von der gleichen Ordnung (*Lactobacillales*) dominiert werden, sind unterschiedliche Gattungen im Menschen (*Streptoccocus*) und Tieren (*Lactobacillus*) präsent.

Zusätzlich beschreibt diese Arbeit erstmals eine schwere Form der apoptotischen Enterokolitis, die durch eine gleichzeitige Behandlung mit Antibiotika und Kortikosteroiden bei kritisch kranken Patienten in der sterilen Umgebung der Intensivstation ausgelöst werden kann. Die Patienten zeigen eine dramatische Verarmung der endogenen GI Mikrobiota, die durch eine starke Reduktion der mikrobiellen Diversität, absolute bakterielle 16S-rRNA-Gene und kurzkettige Fettsäuren (SCFA) gekennzeichnet ist. Zusätzlich kommt es zu einer starken Induktion der CD8+ T-Zellen. Der Krankheit konnten keine spezifischen infektiösen Erreger zugeordnet werden. Stattdessen zeigt sich eine starke bakterielle Fluktuationen in der Mikrobiota der Dickdarmschleimhaut und des Stuhls. Es handelt sich um kommensale Bakterien der Haut bzw. des Rachens, die normalerweise den unteren gastrointestinalen Trakt nicht besiedeln. Dies deutet auf einen Verlust der Kolonisationsresistenz in den Patienten hin. Die Studie zeigt, dass eine einzige fäkale mikrobielle Transplantation (FMT) das

Potential hat, die Symptome zu beseitigen und den Patienten dabei hilft, sich von einer apoptotischen Antibiotika-assoziierten Enterokolitis auf der Intensivstation zu erholen.

Der letzte Teil dieser Arbeit untersucht die Wirksamkeit von wiederholten FMTs bei Patienten mit chronischer, aktiver Colitis ulcerosa (CU), die bereits erfolglos mindestens eine Immunsuppressivum und / oder Anti-TNF-Antikörper Therapie erhielten. In einer offenen kontrollierten Studie unterzogen sich 27 Patienten einer 10-tägigen Antibiotikatherapie. Eine Gruppe der Studie wurde anschließend wiederholt mit FMTs behandelt, die andere nicht. Während die Antibiotika-Kontrollgruppe eine Drop-out-Rate von 50% aufweist und nur 1/10 (10%) der Patienten eine klinische Antwort zeigt, zeigen in der FMT-Gruppe 10/17 (59%) Patienten eine klinische Antwort und 4/17 (24%) eine Remission auf. Zusätzlich identifiziert die Studie eine signifikante Signatur in der Spender-Mikrobiota, die mit klinischen Remissionserscheinungen bei Patienten assoziiert ist. Diese mikrobielle Zusammensetzung unterscheidet sich signifikant von anderen Spenderproben, basierend auf unweighted UniFrac. Sie zeichnet sich durch erhöhte Konzentrationen von *Akkermansia muciniphila*, *Ruminococcus* spp. sowie eine erhöhte Artenvielfalt aus.

Die Wirksamkeit von FMT bei UC-Patienten wird durch die taxonomische Zusammensetzung der Spender-Mikrobiota beeinflusst. Eine spezifische Spenderauswahl bzw. bestimmte mikrobielle Präparate könnte deshalb zu neuen Behandlungen in CU führen könnte.

Abstract

Numerous studies have shown that clinical interventions in the stomach result in alterations of the fecal microbiota, which indicates an important function of the stomach in gastrointestinal (GI) homeostasis. Therefore, in this thesis we gradually characterize the human mucosal and luminal microbiota of the stomach including the adjacent mucosa from esophagus and duodenum using 16S rRNA amplicon sequencing and compare it to the stomach microbiota of mice and gerbils. We found that the luminal microbiota in humans is dominated by *Firmicutes* and *Bacteroidetes*. Nevertheless both fractions show big variations that that are not associated with any health status. The composition of the stomach microbiota depends on the influx from upstream GI locations while selectively enriching (*Rothia mucilaginosa*, *Porphyromonas* and *Lachnospiraceae*) and depleting (*Streptococcus*) specific bacterial taxa throughout the stomach mucosa. The taxonomic composition shows big differences in gerbils and mice compared to humans. While all three are dominated by the same class order (*Lactobacillales*), different genera are present in human (*Streptoccocus*) and animals (*Lactobacillus*). These findings are of significant interest as gerbils and mice represent important models for stomach infectious disease research, especially studies focusing on *H. pylori* and gastric cancer.

In addition, this work shows that concomitant treatment with antibiotics and corticosteroids in critically ill patients in the sterile environment of intensive care units (ICU) can result in a severe form of apoptotic enterocolitis accompanied by a strong induction of $CD8^+$ T-cells. The patients presented a dramatic depletion of the endogenous gastrointestinal microbiota that is characterized by a severe reduction of microbial diversity, absolute bacterial 16S rRNA genes and short chain fatty acids (SCFA). The disease could not be associated to any specific infectious agent. Instead strong fluctuations in the colonic and fecal microbiota determined by skin or oropharynx commensals are, which are usually absent in the lower gastro-intestinal tract, indicate a loss of colonization resistance in these patients. We show that a single fecal microbial transplantation (FMT) has the capability to change this conditions and helps patients to recover from an apoptotic antibiotic-associated enterocolitis in the ICU.

This work also investigates the efficacy of repeated FMTs in patients with chronic active ulcerative colitis (UC), who already had treatment failures for at least one immunosuppressive agent and/or anti-TNF-antibody. UC is associated with dysbiosis of the gastrointestinal microbiota. In this open controlled trial 27 patients received a 10-day antibiotic treatment. While one group of the trial received repeated FMTs afterwards the other patients were not treated with FMT. The antibiotics control group had a drop-out rate of 50% and only 1/10 (10%) patient showed a clinical response, in the FMT-group on the other hand 10/17 (59%) patients showed a response and 4/17 (24%) a remission. Additionally we could identify a signature in the donor microbiota that is associated with signs of clinical remission in patients. The microbial composition from these donors significantly separates from other donor samples based on unweighted UniFrac distance. Its features are high concentrations of *Akkermansia muciniphila*, *Ruminococcus* spp. and an increased richness based on observed species. We could show that the efficacy of FMT in UC patients is influenced by the taxonomic composition of the donor microbiota and that specific donor selection or microbial preparations might lead to new treatments for UC.

1. Introduction

Today we know that the human body is colonized by a variety of microorganisms including protists, fungi, viruses and bacteria. About $3.8*10^{13}$ bacterial cells and between 1000 and 1150 different bacterial species colonize the human gastro-intestinal (GI) tract (3,4). In its entirety it can be subsumed as microbiome, which is often referred to as an organ on its own (5–8) due to its ability of metabolite production as well as being at the same time responsive to and interacting with its organ and the periphery, processes summarized as the gut-brain-axis (9–12) and the gut-lung axis (13–15). Due to its abundance of unique genes and its unique capabilities, the microbiome is also called our second genome (16,17) as the genes of our associated microbes, with an estimation of more than 3 million bacterial genes, outnumber our own human genes by more than a hundredfold (4,18). But how could this extensive amount of data be generated?

1.1. A brief history from culture to database

Modern bacteriology was founded in the golden age of microbiology in the late 1800s and early 1900s, especially by three eminent scientists: Ferdinand Cohn, who established sterile culture mediums, created a new bacterial taxonomy according to physiological characteristics and morphology, including the concept of bacterial species, and being the first who discovered bacterial endospores (19). Louis Pasteur is known for the discoveries of microbial fermentation, pasteurization and the principles of vaccination, including the creation of vaccines against the rabies virus and anthrax infection (20–22). And Robert Koch, who was able to identify the causative agents for cholera, tuberculosis and anthrax, including the creation of Koch's postulates (23,24). Since then the scientific focus in the field of microbiology has been put on pathogens with great success. For example *Helicobacter pylori* and its role in gastritis and peptic ulcer disease (25), but also viruses like the retrovirus human immunodeficiency virus (HIV) that causes HIV infection leading to the acquired immunodeficiency syndrome (AIDS), and many more (26,27). Nevertheless microbiology and identification of certain bacteria and potential causative agents of disease were limited by available

cultivation methods of microorganisms. These limits started to be overcome when pioneers in sequencing RNA and DNA like Walter Fiers, first sequenced a whole RNA gene and later the whole genome of single-stranded RNA virus bacteriophage MS2 in 1976 (28,29). In the following year Frederick Sanger characterized the first full DNA genome from bacteriophage Φ X174 with the dideoxynucleotide chain-termination method (30,31), hitting the next milestone by shotgun-sequencing of the entire bacteriophage λ genome in 1982 (32). Together with the findings, that 16S ribosomal RNA (rRNA) genes differ for each species being a convenient identifier for targeted sequencing (33) the era of culture-independent identification of microorganisms was on the rise. Steady development and refinement of methods led to an increased speed and a decrease of costs for sequencing. The progress towards automated fluorescence based Sanger-sequencing machines from Applied Biosystems, capable of generating 1,000 base pairs (bp) a day (34), initiated the Human Genome Project (HGP). The publicly funded, international consortium was founded in 1990 and announced the complete sequencing of the human genome and finally published the whole human genome sequence on Apr 14, 2003 (35,36). Contemporaneous Craig Venter and his company Celera that sequenced the first whole genome of a free-living and self-replicating organism, the bacterium *Haemophilus influenza* (37), started a privately funded, pairwise end sequencing approach for whole genome shotgun sequencing of the human genome in 1998. They were able to publish the human genome at the same time as the HGP in 2001 (38). In 2005 the first measure for next-generation sequencing (NGS), or high-throughput, platforms succeeded by resequencing of the *Escherichia coli genome* (39) and assembly of *Mycoplasma genitalium* by Margulies et al. and 454 Life Science. The great advantages in this technique were on the on hand increased sequencing speed and on the other hand a tremendous reduction of costs by factor of almost 10 compared to conventional sequencing at that time (40). After the acquisition of 454 by ROCHE the platform dominated the sequencing sector. Using 454 NGS technology the Human Microbiome Project (HMP) was able to sequence 16S rRNA genes from more than 10,000 specimens of healthy human subjects by targeting hypervariable regions (HVR) V3 to V5, providing an in-depth definition of the healthy human microbiome at 18 different body sites (41,42). The deep sampling and cost efficiency outweighed the loss of sensitivity in species

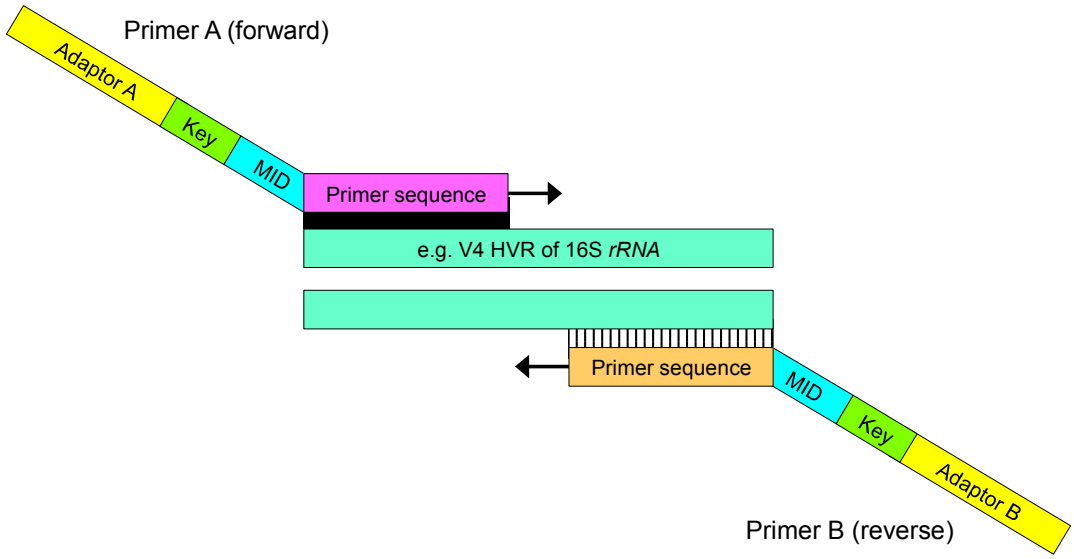
identification, due to rather short reads of about 400 bp produced by 454 sequencing, not covering the entire length of the 16S rRNA gene (about 1.5.kb) leading to potentially ambiguous identification at species or genus level. Even shorter reads with a length of about 25-300 bp are produced by Illumina's MiSeq technology, depending on the Kit that is used. This technology allowed sequencing of a single HVR (17), but deeper sampling at lower costs enabled Illumina's MiSeq as a state of the art sequencing platform for 16S rRNA gene amplicon sequencing used for most microbiome surveys up to now. Thanks to improvement of Illumina chemistry sequencing increased in length up to 500-600 bp using 300bp pair-end sequencing allowing also sequencing of multiple HVRs with Illumina MiSeq (43). Both the 454 and the MiSeq technologies were used for this thesis. The Ion Torrent is an alternative NGS method frequently used for 16S rRNA sequencing. Third generation sequencing methods, like PacBio, SMRT or the Oxford Nanopore, do not include an amplification step during sequencing library preparation, hence they can perform single molecule sequencing in contrast to Illumina or 454. They even produce very high read lengths exceeding 6-8 kb on average, leading to a much higher resolution for microbial identification. Although these systems are more and more used, especially portable systems like the nanopore MinION, due to a relatively high error rate and low output third generation sequencing systems are not able to replace sequencing systems like Illumina yet (44,45).

1.2. 16S rRNA gene as target for amplicon sequencing

The 16S rRNA and 19 ribosomal proteins forge the small subunit (30S) of the prokaryotic ribosome, which is responsible for the translation of messenger RNA (mRNA) into proteins via linkage of amino acids. Together with the large subunit (50S), it forms the entire prokaryotic ribosome (70S) (46). The 16S rRNA is encoded by the 16S rRNA gene. The advantage of selecting the 16S rRNA gene for amplicon sequencing is that the gene contains alternating conserved and up to 9 hypervariable regions (HVRs), scattered throughout the gene, which is of about 1.5 kb in size. Due to its essential biological function, mutations are allowed only in the HVRs, thus it serves as a molecular clock, enabling specific identification of microorganisms (47). In the last years various primers for 16S rRNA gene targeted

amplicon sequencing were designed, complementary to the conserved regions, capturing the majority of bacterial taxa when used in polymerase chain reaction (PCR) based assays. To increase the coverage most used primers include degenerate bases, representing various bases and therefore the forward and reverse primers include multiple copies with different nucleotide sequences. The best applicable set of primers also depends on the sequencing system being used and whether bacteria or archaea should be captured (48). The HMP used for instance the oligonucleotides 27F and 534R amplifying V1-V3, which was also used in this work, or 357F and 926R, for V3-V5 with 454 sequencing (42). Due to reduced size of sequencing fragments generated by the Illumina NGS system, different primers are used compared to 454, especially 515F and 806R, amplifying the V4 region (49), which have also been used by the Earth Microbiome Project (EMP) (50) and in this work (2). Of course there are several other primers used, such as the 341F/518R and 967F/1046R, covering the V3 or V6 region respectively, for the Ion Torrent platform (51). Furthermore the obligatory sequencing adapters and barcodes are incorporated during the PCR using fusion primers (48). The design of the fusion primer depends also on the NGS platform (Figure 1). If Illumina systems are used, fusion primers contain adapters and primer pads of 10 nucleotides (nt) on both the forward and the reverse primer to prevent hairpin formation, and a 12 nt barcode sequence on the reverse primer (48). The sequencing of 16S rRNA with 454 system from Roche is based on one-way reads amplicon sequencing in most studies. The forward fusion primer (Primer A) contains a specific 26 nt long adapter sequence followed by 4 nt key sequence and the multiplex identifiers also called MIDs or barcodes. The reverse fusion primer (Primer B) also contains a specific leading sequence of 26 nt length followed by the 4 nt key sequence. Primer A and B enable directional sequencing. For 16S rRNA gene amplicon sequencing, application of the Lib-L chemistry ensures sequencing from the Primer A end (52). The Ion Torrent uses two different fusion primers. Both with adaptors and only the forward primer containing a 10-12 nt barcode followed by a 6 nt key. In all cases the reverse primer is the same for all PCRs but various forward primer are needed with unique identifiers (barcodes, MIDs) for each sample. The barcodes later allow the sequences to be reassigned to each sample, which is achieved during the de-multiplexing in the bioinformatics pre-processing of the sequences (48).

Fusion primer design for 454 sequencing (incl. schematic representation of PCR reaction)



Fusion primer design for Ion Torrent sequencing

Fusion primer design for Illumina sequencing

Figure 1. Fusion primer designs for targeted amplicon sequencing on different NGS sequencing machines.

1.3. Bioinformatics processing and data analysis

Nevertheless the fast and cheap generation of NGS data has also its disadvantages - the higher error rate in particular. Hence bioinformatics tools for extensive processing of generated reads from systems such as 454 and Illumina's MiSeq are needed to ensure good quality of data used as input for microbiota analysis. State of the art algorithms for 16S rRNA data processing are provided by mothur (53). Mothur is an open source development platform that facilitates bioinformatics analysis for 16S rRNA gene sequences in microbial and ecology

research. It is able to process generated reads and data from several sequencing technologies including 454 and Illumina MiSeq used in this work. Furthermore the platform supports Sanger, PacBio, IonTorrent and Illumina HiSeq. Bioinformatics analyses for this work have mainly been conducted according to their 454 (54) and MiSeq Standard Operating Procedure (SOP) (55) with default settings. Initially sequencing errors are getting reduced using flowgrams, mothurs implementation of PyroNoise or alternatively using the quality or Phred scores from the sequencing run respectively. Furthermore the dataset is simplified by removing redundant sequences, without losing any information to speed up downstream analysis. Subsequently the reads are aligned to a reference database e.g. the SILVA reference alignment (56) and those sequences that do not overlap in the same alignment area are removed, depending on which variable region of the 16S rRNA gene was amplified. Next sequences that only differ in 1 or 2 bases from the most homolog sequence are clustered. These errors mainly originate from sequencing errors, Subsequently chimeras are being removed for example using UCHIME (57). This algorithm detects chimeric 16S rRNA sequences formed during PCR and originating from two different 16S rRNA gene targets. Through classification using the Ribosomal Database Project (RDP) reference files (58), the dataset can be further improved by removing sequences that are not valid for downstream analysis. This includes sequences classified as mitochondria, chloroplasts, unknown sequences that cannot be classified at Kingdom level, and sequences classified as eukaryotic or archaeal 18S/16S rRNAs, originating from unspecific amplification using 16S rRNA gene primers. The required processing for MiSeq data is in general very similar to sequences acquired from 454 sequencing using a different approach reducing PCR and sequencing errors. Illumina's MiSeq platform is using paired-end reads, therefore the forward and reverse reads need to be combined initially. In that way the quality of sequence data is improved, since the ligation of both reads leads to an overlapping region. The algorithm is favoring the bases with a higher quality score and thereby reducing PCR and sequencing errors (53,54).

A widely used strategy to analyze microbial diversity from 16S rRNA gene amplicon sequencing is to calculate operational taxonomic units (OTU) that classify groups of closely related sequences. In general OTUs are defined based

on a threshold of 97% similarity based on Stackebrandt and Goebel (59). Quantitative Insights Into Microbial Ecology (QIIME) (60), another open-source bioinformatics pipeline for microbiome analysis, offers three main strategies for OTU picking by implementation of various open source tools, like SortMeRNA, SUMACLUST and swarm (61–63), or closed-source tools, like UCLUST (64), which has been used for this studies:

- *De novo OTU picking*: In this process the reads are clustered against each other without using any external reference database. This strategy's benefit is that all reads within the datasets are clustered, but it is very slow as it doesn't run in parallel. Therefore this is not suitable for large datasets generated for instance from a full HiSeq 2000 run. This strategy is not applicable for a comparison of non-overlapping amplicons (65).
- *Closed-reference OTU picking*: This approach clusters the reads against a reference database, e.g. Greengenes, with existing clusters and removing all sequences that do not match with any sequences in the collection. This is a very fast approach as it runs in parallel. It allows comparing of non-overlapping amplicons and provides good quality data for trees and taxonomy, as the OTUs are already defined in the reference collection. However a closed reference picking strategy makes it impossible to detect novel microbial diversity as the clusters are restricted to the known references and was therefore not applied in this studies (65).
- *Open-reference OTU picking*: This approach is very similar to the closed-reference picking strategy with one major difference. The reads that do not match to the reference database will be subsequently clustered de novo. In this way novel diversities will be included in downstream analysis compared to the closed-reference approach with the downside of time lag depending on the amount of novel sequences analyzed (65).

Thereby generated OTU sequences are thought to represent a unique correct biological sequence within a dataset that is at least 3% different from other OTU sequences (66). With generated OTU tables and assigned taxonomies microbial ecologists are able to evaluate taxonomic compositions of certain habitats and samples. No cultures are necessary only isolation of the respective DNA, with

subsequent amplification and sequencing of the 16S rRNA genes. Furthermore this data can be used to compute the alpha diversity, the habitats mean species diversity, or beta diversity, the difference between the species diversity from two or more separated microbial communities or groups (67). The alpha, or within-sample, diversity can be calculated by various metrics, describing the species richness and/or evenness within a single sample. Often used metrics are "observed species" and "chao1", indicating richness, the "Simpson index", a parameter of dominance or evenness within a community, and the "Shannon" or "Shannon-Weaver index", which incorporates both components of biodiversity (richness and evenness) (68). Additionally beta, or between-sample, diversity is a useful measure to compare different microbial communities. The beta-diversity is calculated by different distance or dissimilarity metrics and mainly presented as Principle Coordinates Analysis (PCoA) or hierarchical clustering. Frequently used distance metrics are the "unweighted" and "weighted UniFrac" distance, first being a qualitative measure using presence or absence information of OTUs to reveal community differences and second a quantitative measure by incorporating the relative abundance of taxa (69,70). The "Bray-Curtis index" is also frequently used to describe dissimilarities between different communities given as inverted ratio of shared taxa and the sum of all taxa in all communities. Hence a "Bray-Curtis dissimilarity" of 0 means the community is qualitatively identical and 1 indicates that both communities do not share a single species (71).

1.4. From data sequencing to applied research

Microbiome research benefits from this technological and bioinformatics evolution. Hence it changed the focus in microbiology away from investigation of singular pathogenic organisms to pathogenic communities and the microbiota as a whole. Research started to investigate the microbiota that inhabits the human body especially under healthy conditions to understand the interactions between "them and us". These interactions are manifold. For instance, the majority of our gut microbes are either harmless or beneficial for our health and thereby protect us from enteropathogens by forming an intestinal colonization resistance (72–74). They also help us to extract energy and nutrients from our diets (6,75–77), they can change our bile acid and choline metabolism (78,79) and they are essential for

the development of our immune system (80). It could be shown that the microbiota is able to induce induction of colonic regulatory T-cells (Tregs) (81) as well as T helper (Th) 17 cells (82). Interestingly both cell types seem to be involved in the pathogenesis of certain autoimmune diseases and inflammatory bowel disease (IBD) (83,84). The gut microbiota, including their immunostimulatory signals and metabolites, directly and indirectly influences the generation and expansion of Th17 cells and intestinal Tregs. Therefore therapeutic approaches that modify the intestinal microbiota and alter dysbiosis, for instance exogenous administration of commensal bacteria, could influence and regulate the susceptibility to autoimmune diseases and IBD (85,86). Short-chain fatty acids (SCFAs), like butyrate, are bacterial metabolites produced by fermentation of dietary fibers, are able to induce differentiation of Tregs (87). Interestingly it could be shown that on the one hand the butyrate receptor monocyte chemotactic protein-1 (MCP-1) is down-regulated in the colonic mucosa and on the other hand mean concentrations of butyrate and propionate levels are reduced in stool samples of IBD patients compared to healthy subjects (88,89). Additionally SCFAs have anti-inflammatory capacities via G protein-coupled receptor 43 (GPR43) signaling in the colonic epithelium (90).

There are various factors that are able to directly modulate our gut microbiota, such as diet. The consumption of a certain diet is associated with certain enterotype classifications of the human gut microbiota. While the three enterotypes, that are characterized by high levels of either *Bacteroides* (type1), *Prevotella* (type 2) and *Ruminococcus* (type3), show no associations with age, body weight, gender or nationality (91), diet seems to be a driving factor shaping the gut microbiota. It could be shown that high uptake of proteins and animal fat, typical for Western diet, is associated with enterotype 1. Whereas a nutrition high in plant-derived complex carbohydrates, that is predominant for instance in rural Africa, is associated with enterotype 2 (92,93). But also food supplements, probiotics and vitamins, e.g. high doses of Vitamin D3, have been reported to shape microbiota composition (94,95) as well as a hygiene practices (96) and drug therapies, especially antibiotic treatment. The ladder eventually leading to serious health complications as presented in the course of this work (1).

In general a balanced microbiota is very important for homeostasis of the GI tract. Disruption of this system leads to imbalances of taxonomic abundances a condition termed dysbiosis. Dysbiosis is reported to be associated with several diseases, such as IBD (97,98) and multiple sclerosis (MS) (99), in both of which reduced levels of SCFAs and Tregs can be found (100,101). But also metabolic and central nervous system (CNS) related disorders, like obesity (102), type 1 and type 2 diabetes (T1D, T2D) (103,104) or autism spectrum disorder (ASD) (105) have been associated with taxonomic shifts in the intestinal microbiota (106). Nevertheless changes in the taxonomic compositions always need to be questioned since they could either be a cause or a consequence of a given disease.

1.5. FMT in recurrent *Clostridium difficile*

FMT is a very prominent and effective way to induce restoration of the GI microbiota, especially in recurrent *Clostridium difficile* infections (CDI) (107). CDI is caused by the pathobiont *C. difficile*, that asymptomatically colonizes most infants but leads to severe pseudomembranous colitis in humans as a consequence of preceding antibiotic therapy (108,109). During infection *C. difficile* proliferates and colonizes the large intestine causing severe inflammation by releasing two protein exotoxins TcdA and TcdB. These toxins lead to colonocyte death, neutrophilic colitis and loss of intestinal barrier function (110). In healthy individuals *C. difficile* is normally not a problem but when patients suffer from reduced colonization resistance in the gut due to treatment with antibiotics, *C. difficile* is able to outgrow other commensals and causes harm. Antibiotic therapy using metronidazole and vancomycin or fidaxomicin are used as treatment against acute infection with *C. difficile*. With a success rate of 50% antibiotic therapy is quite efficient for acute CDI (111). This is not the case for recurrences. Fortunately in 2013 it could be shown that the transfer of healthy donor feces via duodenal infusions leads to resolution of *C. difficile*–associated diarrhea in more than 80% of cases opening the avenue for FMT as a therapeutic approach (107). While FMT has become the first line therapy for recurrent CDI, more and more studies are conducted to use FMT for other GI diseases such as ulcerative colitis (2,112–116). Moreover,

severe dysbiosis recognized in several critically ill patients in the intensive care unit might be treated by FMT (1,117,118), as shown in this work.

1.6. Specific aims

This work is structured in three main parts addressing different questions regarding the dissertation's topic "*The origin and loss of the human gastro-intestinal microbiota integrity and its reconstitution by fecal microbiota transplantation*":

- **Biogeography of the stomach microbiota in humans, mice and gerbils indicates selective enrichment and depletion of specific bacterial taxa**

  Aim: Better microbial characterization of the upper GI-tract microbiota, including the stomach, which has not been covered by the HMP (18), in humans and research related animal models, using 16S rRNA gene amplicon sequencing. The upper GI tract represents the "entry" point of microbes to the lower GI tract and might be a bottleneck for microbial selection, colonization and distribution.

- **Antibiotic-associated apoptotic enterocolitis in the absence of a defined pathogen: The role of intestinal microbiota depletion (1)**

  Aim: Characterizing microbial and host factors under conditions of severely disturbed microbiota-GI homeostasis (severe antibiotic associated enterocolitis in the critical ill) and the effect of microbiota reconstitution by single-FMT, using 16S rRNA gene amplicon sequencing, immunophenotyping and metabolic analyses, in a case series with three individuals.

- **The taxonomic composition of the donor intestinal microbiota is a major factor influencing the efficacy of fecal microbiota transplantation in therapy refractory ulcerative colitis (2)**

  Aim: Discern a microbial signature associated with anti-inflammatory action of repeated-FMT to treat therapy refractory, chronic active ulcerative colitis (UC). Investigate the potential for reconstructing the GI microbiota in UC patients with concomitant dysbiosis (98), using 16S rRNA gene amplicon sequencing, immunophenotyping, in an open prospective trial.

2. Biogeography of the stomach microbiota in humans, mice and gerbils indicates selective enrichment and depletion of specific bacterial taxa

2.1. Introduction

The human stomach was long thought to be sterile, as it acts as first line of defense against orally swallowed pathogens due to its acid production. But Marshall and Warren initiated a paradigm shift with the discovery of the gram-negative and helically shaped bacterium *Helicobacter pylori* and the link to gastritis, ulcer disease as well as gastric cancer opened a new era in gastric microbiology (25). In the last years a variety of studies linked specific microbiota conditions of the GI tract to different metabolic disorders (119–121), the risk for and recovery from infectious diseases (122–125) and immunological diseases, including autoimmunity and allergies (126–128) as well as malignant diseases (129,130).

Despite growing evidence for the significant role of the stomach microbiota for homeostasis of the entire GI tract, there is still a lack of knowledge regarding microbiota dynamics within the stomach and the upper GI tract. Also the HMP did not include the stomach in the microbiome surveys of healthy individuals (18). A limiting factor for sufficient studies of the upper GI tract is caused by limited accessibility of samples, whereas the sampling of fecal specimen is straightforward. Studies with Roux-en-y gastric bypass surgeries showed that GI reconfigurations alter the microbial ecology in fecal samples of mice (131), similar findings could also be shown in humans (132,133). To gain more knowledge regarding the taxonomic bacterial biogeography of the upper GI tract we decided to perform a stratified 16S rRNA gene amplicon sequencing study from upper GI biopsies, including various locations of the stomach (cardia, corpus/fundus and antrum) as well as upstream and downstream adjacent locations from esophagus and duodenum. Additionally we wanted to analyze the luminal content of the stomach. Therefore we sampled gastric aspirates from the same patients and also from mice and gerbils to compare the human with animal gastric microbiota from

models used for stomach infectious disease research, especially studies focusing on *H. pylori* and gastric cancer (134–137).

This project was carried out as cooperation with Prof. W. Florian Fricke at the University of Hohenheim, Stuttgart including a half-year research stay. The results were obtained together with Cynthia Maddox, Christoph Högenauer, Bettina Halwachs, Ute Harrison, Thomas Blanchard, Rainer Haas, Gregor Gorkiewicz and W. Florian Fricke.

A manuscript with the running title "Biogeography and organization of the upper gastrointestinal microbiota in humans, mice and gerbils" has been submitted at the time of thesis publication.

2.2. Materials and Methods

2.2.1. Human study cohort and sample collection

GI biopsies and aspirate samples were collected at the Medical University Graz, Austria from individuals, who underwent upper endoscopy for various clinical indications (Table 1). The Institutional Review Board of the Medical University of Graz approved the study under protocol number EK 23-212 ex 10/11 and all subjects provided their written informed consent to participate in the study. Endoscopic and histologic reports indicated no or minimal evidence of GI pathology in 12 patients, *H. pylori*-associated gastritis and autoimmune gastritis in two patients. One patient had Crohn's disease. Biopsies were obtained by routine esophagogastroduodenoscopy by a gastroenterologist. The order in which samples were taken: 1st) duodenum, 2nd) gastric fluid, 3rd) antrum, 4th) corpus, 5th) cardia, 6th) esophagus. Biopsy samples were immediately transferred to RNAlater, gastric aspirate samples diluted 1-fold in RNAlater and stored at -80°C. Samples were shipped on dry ice to the Institute for Genome Sciences, at the University of Maryland, School of Medicine, Baltimore for sample processing and sequencing. All samples were stored at -80°C until processing.

2.2.2. Animal sample collection

C57BL/6 mice were ordered from the Jackson Laboratory, Bar Harbor, ME, and used to extract stomach samples within five days after delivery at the University of Maryland, Baltimore. Mongolian gerbil stomach samples were collected at the Max von Pettenkofer-Institute in Munich, Germany. Samples from ten mice and ten gerbils were collected as follows: Animals were euthanized, complete stomachs extracted, cut in half at the *curvatura gastrica major* and immediately transferred to RNAlater. Gerbil samples were shipped at room temperature to the University of Maryland, Baltimore. All samples were stored at -80°C upon arrival at the Institute for Genome Sciences, University of Maryland, Baltimore, until processing.

2.2.3. DNA extraction, 16S rRNA PCR and sequencing

Metagenomic DNA was isolated from all samples using a previously described protocol, which includes both enzymatic digestions (lysozyme, mutanolysin, lysostaphin, proteinase K, and RNase) and mechanical disruption by bead beating (138). Briefly, stomach and biopsy samples were vortexed at full speed for three minutes and the entire fluid transferred to new tubes for enzymatic and mechanical lysis. HVRs V1–V3 of the bacterial 16S rRNA gene were amplified using barcoded primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') (139) and 534R (5'-ATTACCGCGGCTGCTGG-3') (140) with Roche/454 adaptors and purified amplicon mixtures sequenced at the Institute for Genome Sciences, University of Maryland, using 454 primer A and protocols recommended by the manufacturer (Roche, Branford, CT, USA), as described previously (141). Raw sequence files have been deposited in the European Nucleotide Archive under the primary accession number PRJEB11744 (secondary accession number ERP013156).

2.2.4. Microbiota analysis and statistical methods.

Raw sequence data from the Roche/454 GS FLX Titanium platform were processed with mothur (53) according to the SOP for 454 sequence data (54) with additional removal of singletons. Subsequent OTU-based microbiota analyses were performed in QIIME v1.8.0 (60), including OTU clustering with 97% similarity using a de-novo OTU picking strategy, rarefaction to a sampling depth of 1415

(human aspirate, gerbil and mouse samples) or 880 (all human and animal samples) reads per sample, and alpha- and beta-diversity analyses. Differences in taxonomic microbiota compositions between groups were analyzed according to linear discriminant analysis effect size (LEfSe) a tool developed by the Huttenhower group (142). Specific statistic tests to determine significance are listed in figure legends. P-values below 0.05 were considered significant ($p<0.05$=*; $p<0.01$=**; $p<0.001$=***). Unless indicated differently, mean values are presented together with standard error of mean (SEM).

2.3. Results

2.3.1. Clinical diagnosis and sample stratification

For a defined analysis of the spatial microbial organization of the upper GI tract mucosal biopsies and aspirates, originating from the stomach and adjacent locations, were collected and 16S rRNA gene amplicon sequencing was performed subsequently. Patients that were included into the study had certain GI pathologies or were asymptomatic and underwent surveillance examinations. Endoscopic and histological examinations found evidence for GI pathologies in 12 patients. Two patients showed autoimmune (Type A) Gastritis, two patients showed *H. pylori* associated gastritis and one had Crohn's disease (Table 1).

Table 1. Indications and symptoms of the patients that underwent upper endoscopy and provided biopsies and aspirate samples.

# IDs	Indication for endoscopy	Symptoms	Clinical diagnosis stomach	Histology final diagnosis
P01	Expectoration of blood	Expectoration of blood	Normal	GERD, normal stomach
P02	Suspected Crohn´s disease	Abdominal pain	Crohn´s disease	Crohns disease
P03	Screening examination	None	Normal	Normal
P04	Dyspepsia	Dyspepsia, bloating	Normal	Normal
P05	Diarrhea	Chronic diarrhea	Normal	Normal
P06	Diarrhea	Chronic diarrhea	Normal	Normal
P07	Dyspepsia	Dyspepsia, bloating	Normal	Normal
P08	Anemia	Anemia	Type A gastritis	Type A gastritis
P09	Suspected submucosal tumor	Epigastric pain	Normal	Normal
P10	Tumor screening	Unexplained weight loss	Normal	Normal
P11	Dyspepsia	Epigastric pain	Normal	Normal
P12	Epigastric pain	Epigastric pain	Normal	Normal
P13	Epigastric pain	Epigastric pain	HP gastritis	HP Gastritis
P14	Epigastric pain	Acute epigastric pain	HP-associated gastric ulcer	HP Gastritis
P15	Screening examination	None	Normal	Normal
P16	Epigastric pain	Epigastric pain	Normal	Normal
P17	Screening examination (type A gastritis)	None	Type A gastritis	Type A gastritis

Representative histology pictures and the different epithelial structures from each location are shown in Figure 2. The esophagus consists of a stratified squamous epithelium. The green arrow is indicating the gastro-esophageal junction between the esophagus and the stomach. The histology indicates the immediate change from a stratified squamous epithelium to the columnar epithelium of the stomach, starting with the cardia, harboring also cardiac glands. Only few cardiac glands are distributed throughout the mucosa of the cardia to primarily secrete mucus. Additionally samples were taken from the corpus, consisting of a simple columnar epithelium with oxyntic glands that secrete hydrochloric acid (HCl), and from the antrum, where also pyloric glands are located, producing mucus and secreting gastrin. The duodenum is also covered by a simple columnar epithelial lining. It includes enterocytes for water uptake and mucin producing goblet cells, as well as Paneth cells in crypts for the production of antimicrobial peptides (AMP). The surface architecture is composed of villi and crypts which is typical for the small intestine.

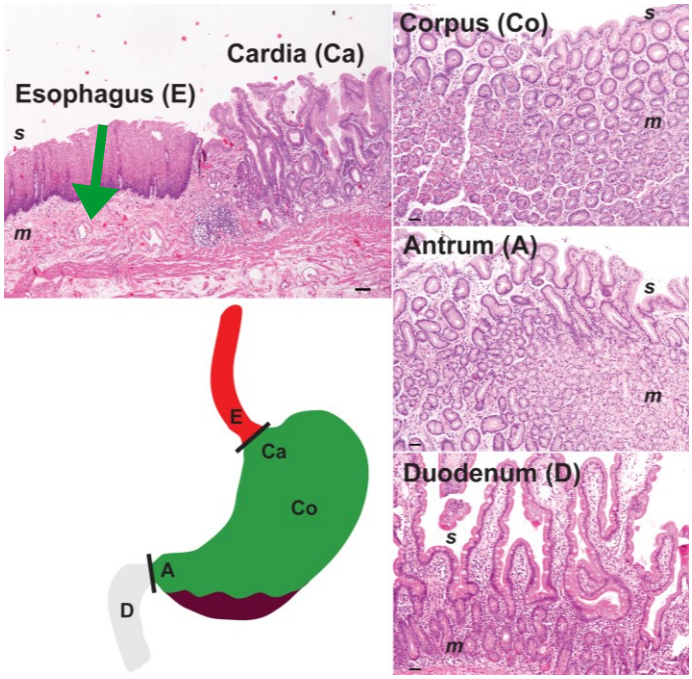


Figure 2. Sampled locations and histology of the stomach and adjacent GI tract. The represented histology shows the junction between the stratified squamous epithelium of the esophagus (E) and columnar epithelium of the gastric cardia region (Ca), the corpus (Co) mucosa including oxyntic gastric glands, and antrum (A), both parts of the stomach, showing mucoid glands in the mucosa. The duodenum (D) has a typical small bowel-architecture with villi and crypts. Abbreviations: s, surface; m, mucosa; magnifications 40x (E, Ca); 100x (Co, A, D).

2.3.2. Microbiota characterization of the upper GI tract

The generated reads from 16S rRNA gene sequencing were processed in mothur and QIIME as described in the material and methods section. All reads were rarified to a sampling depth of 880 reads/sample for all human biopsy and gastric aspirate samples. There was no significant difference regarding read and OTU counts between biopsy samples analyzed at maximum sequencing depth or the rarefied depth. Aspirates show a slightly increased amount of processed and generated reads compared to biopsies, leading to an increased count of OTUs in aspirates. This difference can be offset by even rarefication of reads to 880 reads per sample. The rarefication is needed to compare all samples based on the same sequencing depth. 880 reads represents the minimum read count within the used dataset from esophagus mucosa sample of P02 (Figure 3).

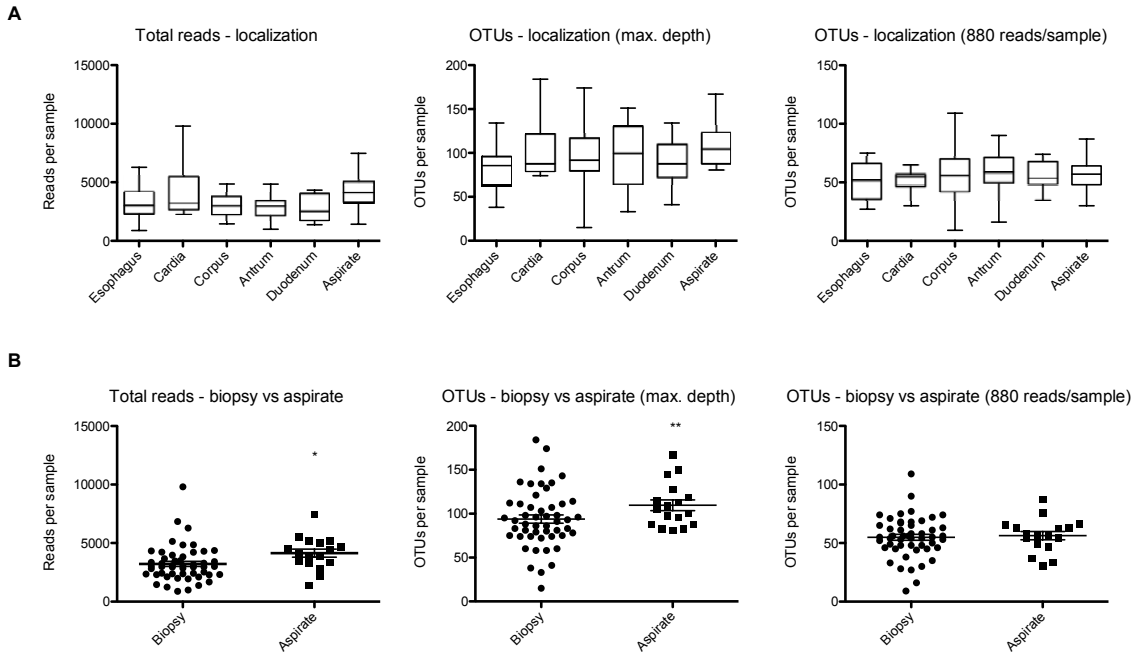


Figure 3. Distribution of generated reads and OTUs used for data analysis of human biopsy and aspirate samples. The total reads and OTUs per samples were compared according to the respective sampling localizations from the upper GI-tract (A), mucosal or non-mucosal origin (B) No significant difference was found for the comparison of total generated reads and OTUs (max. depth and evened to 880 reads per sample) between different localizations. Stomach aspirates show an increase of reads compared to mucosal biopsies. OTUs are significantly increased in aspirates at max. depth but show no significant difference at the applied rarefication depth of 880 reads per sample (Applied nonparametric tests: A:

Kruskal-Wallis test with Dunn's multiple comparison test; B: Mann Whitney test; *p<0.05, **p<0.01, ***p<0.001).

Principle coordinate analysis (PCoA) according to various different distance metrics (Unweighted UniFrac p=0.001, R=0.5181; Weighted UniFrac p=0.001, R=0.4418; Bray Curtis distance p=0.001, R=0.6283) showed that all analyzed samples, including biopsies and aspirates, cluster by individual. No significant clustering according to sample type or location was evident (Figure 4). Stomach samples from *H. pylori* gastritis, were excluded from this analysis.

The overall microbiota from all samples including all patients and locations, but excluding the samples from *H. pylori* positive patients (P13 and P14), was predominated by the phylum *Firmicutes* (73.2 +/- 1.2 %), *Bacteroidetes* (12.5 +/- 0.9%), *Actinobacteria* (9.4 +/- 0.6%) and *Proteobacteria* (2.9 +/- 0.4%). Additionally the taxonomic representation in Figure 5 shows a trend of a constant decrease of *Lactobacillales* on order level from esophagus to antrum.

In samples from patients that were positive for *H. pylori*, microbiota was dominated by this pathogen and analysis indicated the preferred niche for the spiral- or helical-shaped stomach adapted *Proteobacterium*. While the relative abundance of *H. pylori* assigned reads is below 5% in stomach aspirates and below 0.5% in esophagus samples, it goes up to 22% in cardia, the connective region between esophagus and stomach, and even increases over 95% rel. abundance in the corpus and antrum of patients (Figure 6). The duodenum of *H. pylori* positive patients is not presented because biopsies were not sampled from those individuals.

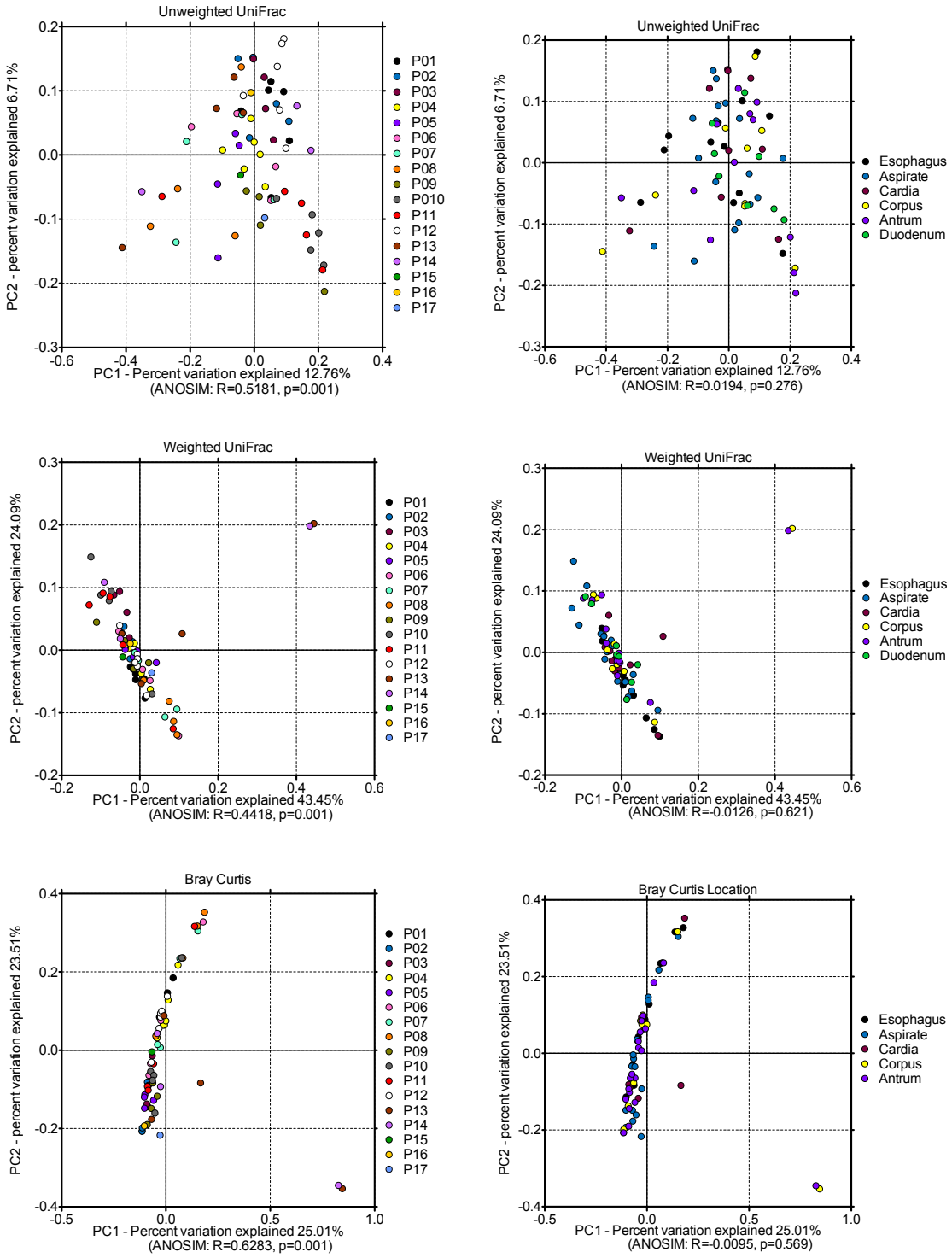


Figure 4. Microbiota analysis of esophagus, stomach (cardia, corpus, antrum, aspirate) and duodenum. Microbiota comparison of *H. pylori*-negative biopsy samples based on taxonomic distance (unweighted UniFrac, weighted UniFrac, Bray Curtis) showing significant differences between patients (left) but not sampled locations (right).

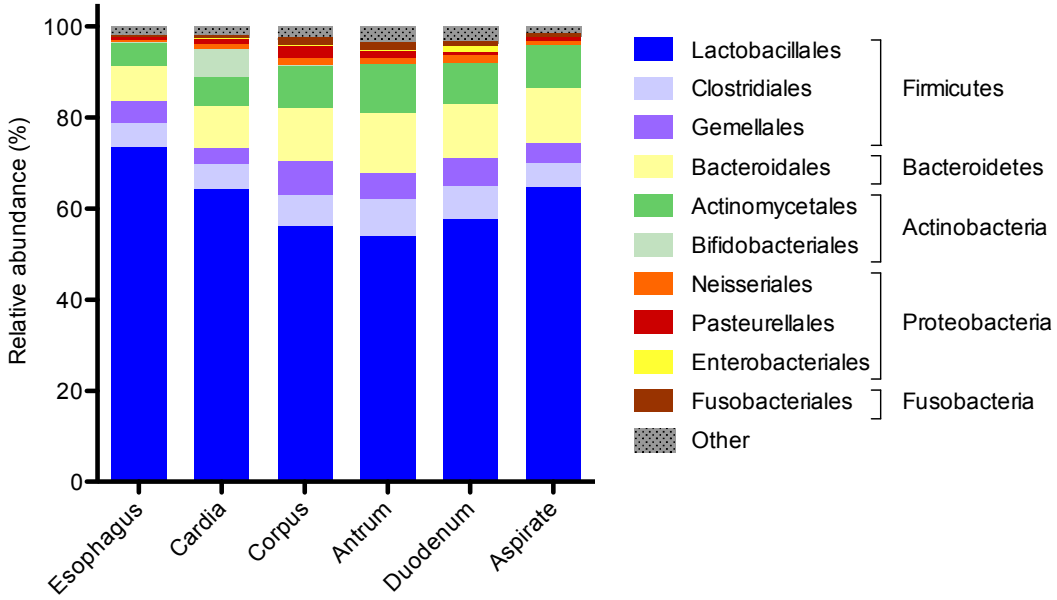


Figure 5. Taxonomy representation of order (left column) and phylum levels (right column). Mean relative microbiota compositions of *H. pylori*-negative biopsy samples, including all OTUs with ≥1% relative abundance in at least one sample.

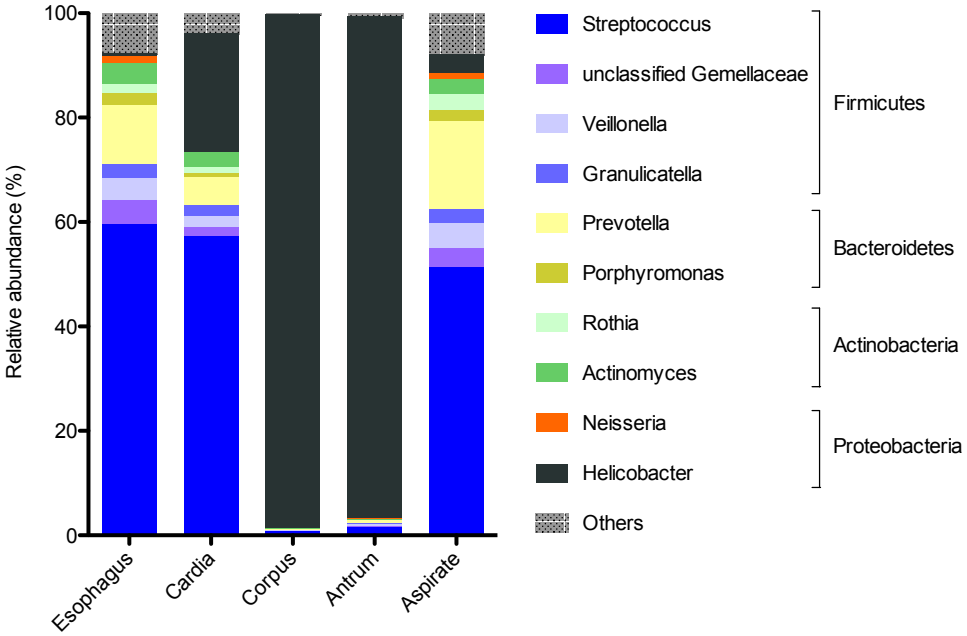


Figure 6. Mean relative microbiota compositions in *H. pylori*-positive samples. The histogram shows the taxonomic classifications at the genus and phylum levels of all OTUs with ≥1% relative abundance in at least one sample.

In summary the analysis of the microbiota of the upper GI tract microbiota shows significant differences in the inter-individual microbiota composition but no significant clustering for different sampling locations.

2.3.3. Differences between esophagus and stomach microbiota

In general the microbial richness in stomach biopsies was significantly increased based on Shannon and Simpson index compared to esophagus but duodenum showed no difference compared to stomach (Figure 7).

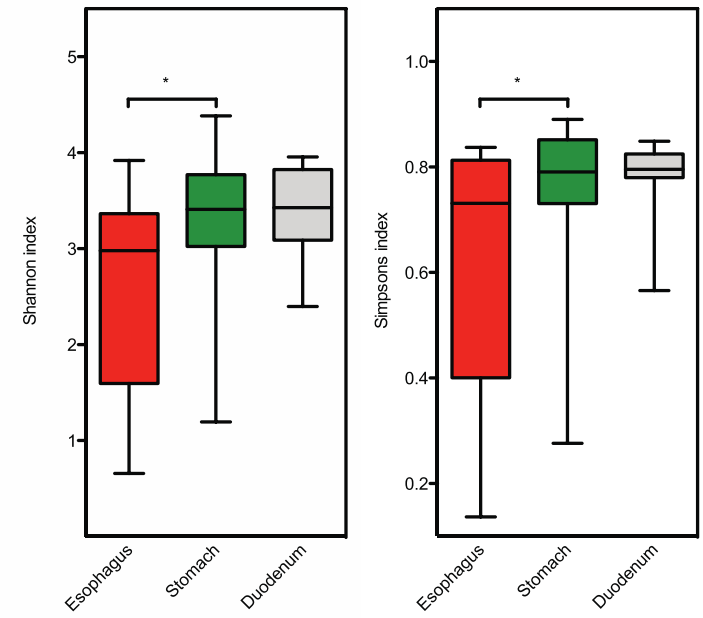


Figure 7. Microbial diversity comparison using Shannon and Simpson indices. Significance determined based on a non-parametric two sample t-test, using 999 Monte Carlo permutations and Bonferroni correction for multiple comparisons (*p<0.05).

Stratifying stomach samples into different locations and also comparing them to stomach aspirates, microbiota analysis showed no significant differences in microbial diversity within the stomach based on equitability, Shannon and Simpson index (Figure 8).

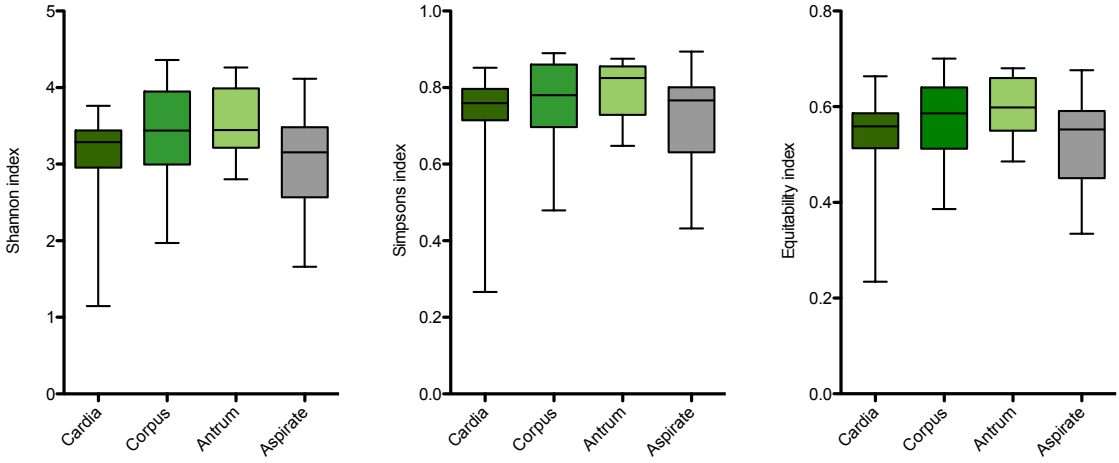


Figure 8. Alpha diversity comparison of the gastric microbiota by location and sample type. Samples were compared based on Shannon and Simpson diversity indices and equitability, as implemented in in QIIME. No significance ($p<0.05$) was found for the comparison of any two sample types and alpha diversity parameters, based on a non-parametric two sample t-test, using 999 Monte Carlo permutations and Bonferroni correction for multiple comparisons.

Next we sought to identify different taxa that may contribute to the microbial shift from esophagus to stomach calculating linear discriminant analysis (LDA) scores to differentiate significant different taxa using LEfSe analysis. Applying a threshold of an LDA score of 2.0 or higher, four different taxonomic lineages with a relative abundance higher than 0.1% could be identified as a discriminatory factor. Whereas an increase of *Firmicutes* / *Bacilli* / *Lactobacillales* / *Streptococcaceae* / *Streptococcus* and unclassified *Streptococcus* was associated with esophagus microbiota, an increase in *Actinobacteria* / *Actinobacteria* / *Actinomycetales* / *Micrococcaceae* / *Rothia* / *mucinlaginosa, Gemella* / unclassified *Gemella, Porphyromonas and Lachnospiraceae* could be determined in stomach biopsies (Figure 9).

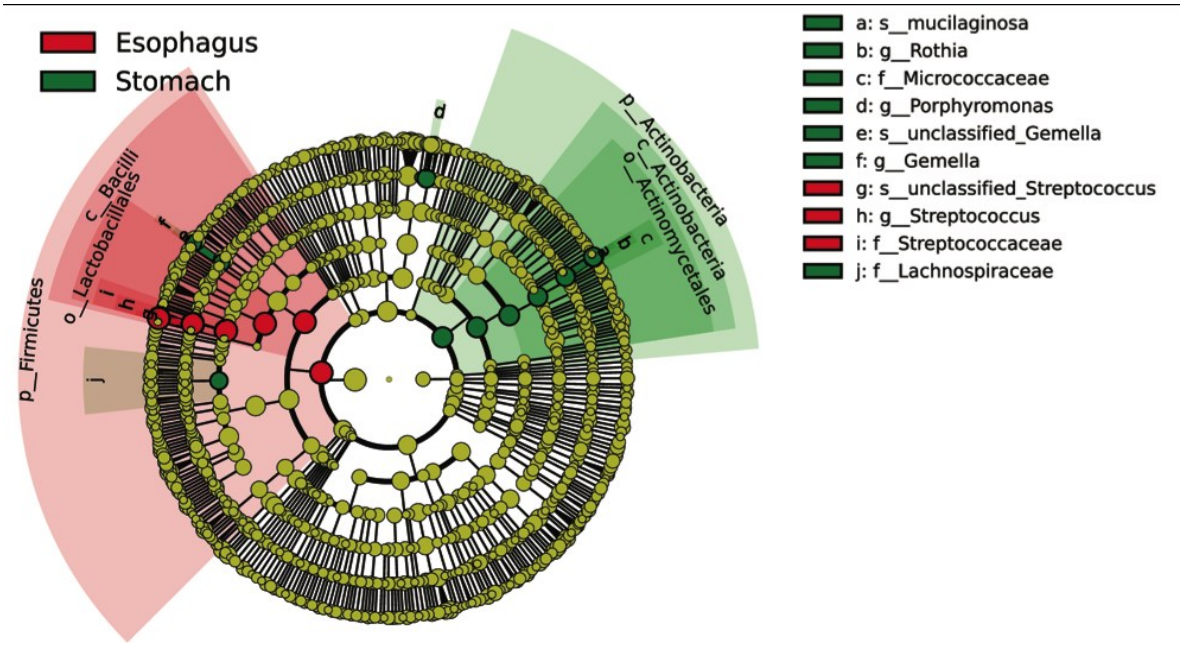


Figure 9. Bacterial taxa with differential relative abundance between *H. pylori*-negative esophagus and stomach biopsy samples. Taxonomic differences have been calculated using a linear discriminant analysis effect size of >2.0 as determined with LEfSe.

By comparing stomach and duodenum mucosal samples, we identified only one discriminant taxon. The relative abundance of *Veilleonella* sp. (mean rel. abundance <0.1%) is significantly enriched in stomach mucosa (Figure 10).

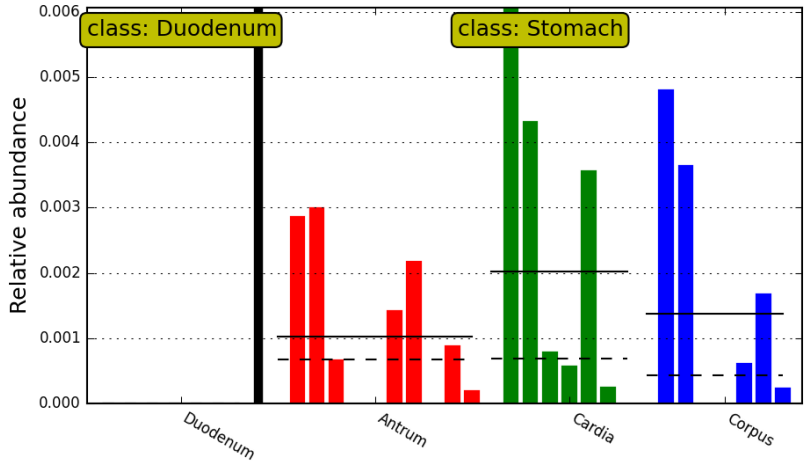


Figure 10. Discriminative taxa between mucosal stomach and mucosal duodenum microbiota. *Veillonella* sp. was significantly enriched in the microbiota of stomach mucosa samples regardless of the location within stomach. LEfSe, LDA > 2.0.

Interestingly all discriminative bacterial taxa between esophagus and stomach microbiota, showed gradual shifts in concentration based on sample location from cranial to caudal with the following order: 1st esophagus, 2nd cardia, 3rd corpus and 4th antrum. According to a Spearman's rank test the relative abundance of *Streptococcus* spp. showed a negative correlation in downstream samples (R=-0.46, p=0.004) whereas *R. mucilaginosa*, *Porphyromonas* spp. and *Lachnospiraceae*, the three taxa that were enriched in stomach, showed a positive correlation (R=0.40-0.41, p<0.02). The strong correlation that was present for all four taxa slightly decreased when duodenum samples were included into the analysis. This might be an indicator that the stomach is representing a selective ecosystem enriching or depleting a certain type of microbes which has an effect on lower segments of the GI tract (Figure 11).

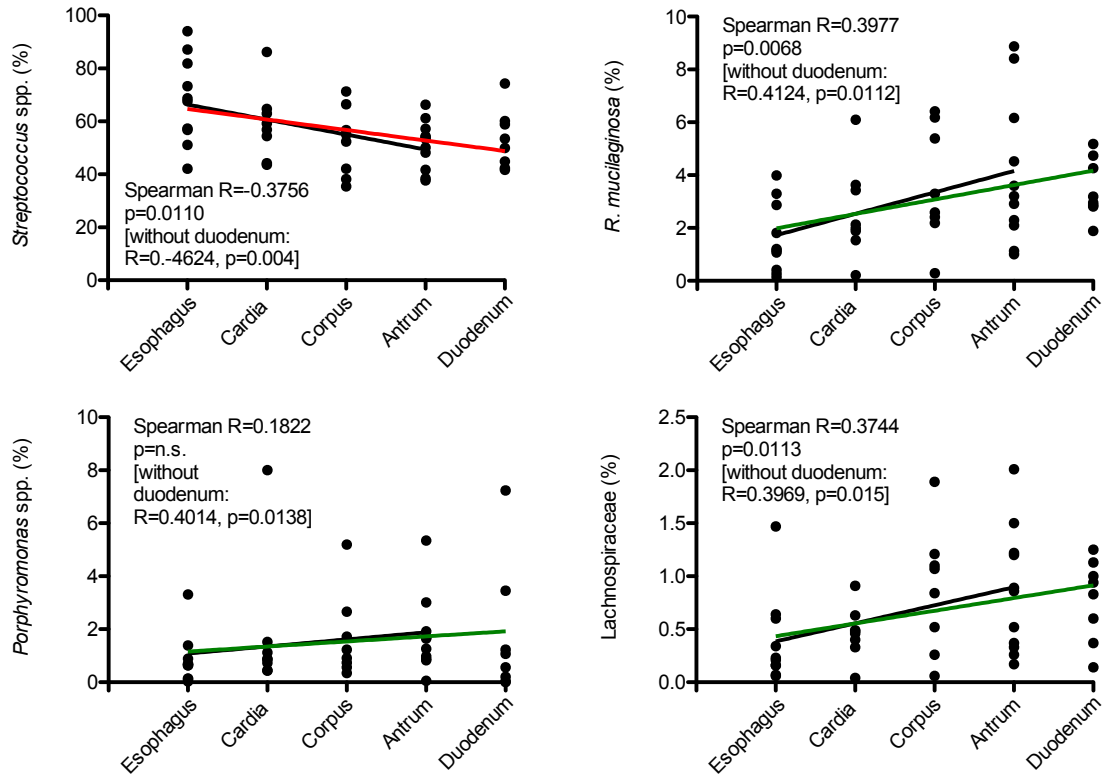


Figure 11. Shifts in relative abundance of differentially abundant taxa from esophagus to stomach and duodenum. Relative abundances of bacterial taxa with a mean of >0.1% and an LDA >2.0 (LEfSe) of all stomach biopsies combined against esophagus samples were plotted along the x-axis of the descending upper GI tract. Correlations and corresponding p-values were determined separately with (black lines) and without (red lines) duodenal biopsy samples, using non-parametric Spearman's rank correlation coefficient analysis (two-tailed).

To sum up the differences between the microbiota of esophagus and stomach, a significant enrichment of microbial diversity in stomach compared to esophagus was evident. All taxa, *Streptococcus* spp., *R. mucilaginosa*, *Porphyromonas* spp. and *Lachnospiraceae* that show a significant different relative abundance in esophagus compared to stomach mucosa display a gradual increase or decrease of relative abundance following the anatomy of the GI tract.

2.3.4. Gastric aspirate microbiota

As already shown in Figure 8 there was no significant difference in microbial diversity between gastric juice and mucosal biopsies, but the two dominant phyla *Firmicutes* and *Bacteroidetes* together were significantly enriched in aspirates compared to mucosa from the stomach (Figure 12).

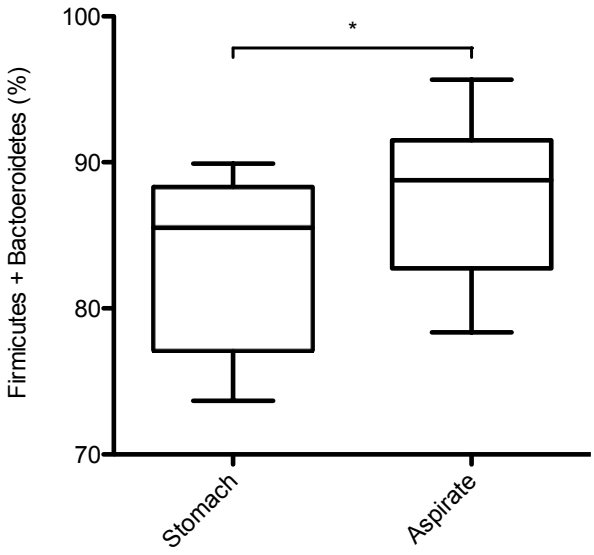


Figure 12. Inter-individual variations in gastric aspirate relative microbiota compositions. (A) Comparison of combined fractions of *Firmicutes* and *Bacteroidetes* in gastric aspirate samples compared to biopsy samples using non-parametric Mann Whitney U test (*p<0.05).

Correlation analysis of weighted UniFrac distances from single human aspirates to all human esophageal biopsies, with relative abundances of *Firmicutes* and *Streptococcus* spp. from the respective aspirate sample, showed a significant correlation based on non-parametric Spearman rank's coefficient analysis. The higher the rel. abundance of *Firmicutes* or *Streptococcus* the more similar the

gastric aspirate microbiota is to the esophageal microbiota. This positive correlation indicates that the similarity of stomach and esophagus microbiota is depending on the concentrations of *Streptococcus* in the stomach. Hence inter-individual differences in rel. abundance of *Firmicutes* and *Streptococcus* could be a consequence of temporal variations in bacterial influx from upstream locations (e.g. oral cavity) (Figure 13).

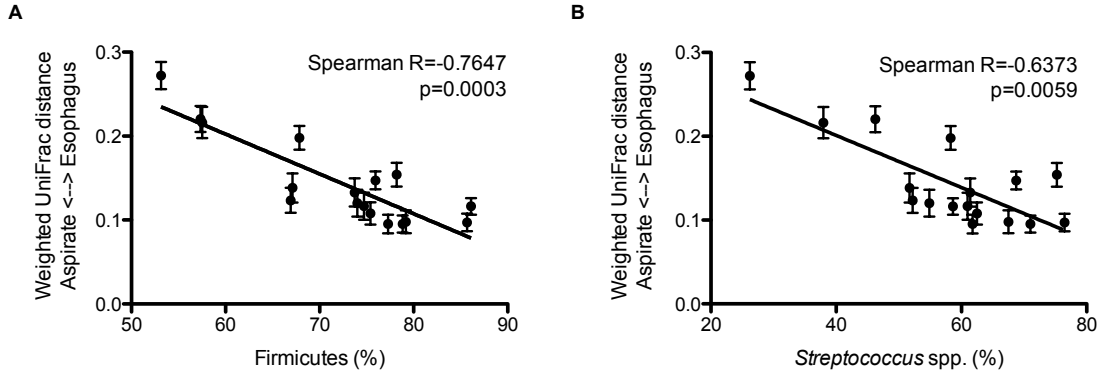


Figure 13. Similarity of stomach and esophagus microbiota. Correlation of taxonomic distance between gastric aspirate and esophageal biopsy samples (weighted UniFrac) with the relative abundance of the phylum *Firmicutes* (A) or the genus *Streptococcus* (B) in aspirate samples, based on Spearman's rank correlation coefficient analysis (two-tailed). Each bar shows the range of taxonomic distances found between an individual gastric aspirate sample and all esophagus biopsy samples, as well as the relative abundance of *Firmicutes* or *Streptococcus* of the aspirate sample.

The concentration of both dominant taxa *Firmicutes* and *Bacteroidetes* was actually highly fluctuating in gastric aspirates between individuals (*Firmicutes*: 53.1 - 86.1%; *Bacteroidetes*: 0.2 - 35.5%). Interestingly we identified the same degree of variation in both dominant phyla in gastric aspirate samples from another recently published study (138). The variation gradient between *Firmicutes* and *Bacteroidetes* in both studies was independent from the patients' health status (Figure 14).

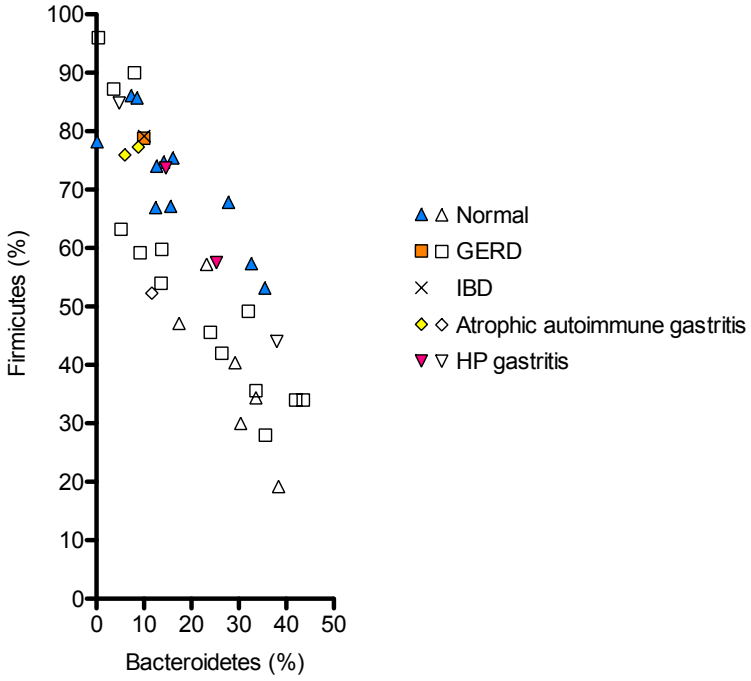


Figure 14. Variation in the relative abundance of *Firmicutes* and *Bacteroidetes* among gastric aspirate samples. This figure shows merged data from our study, represented by colored symbols, and from a previous study from Rosenvinge et al. (138). The symbols are based on patient health background. Numerical data from Rosenvinge et al (138) was reanalyzed for this work. The data was graciously provided by corresponding author Prof. W. Florian Fricke.

We additionally tried to identify the predominant phyla in the bacterial 16S rRNA transcripts, representing the protein synthesis potential and generally indicating active microbes (143). Therefore we used concomitant 16S rRNA gene data and 16S rRNA transcript data generated via reverse transcriptase PCR also published by Rosenvinge et al. (138). Based on relative abundance we found a significant increase in *Bacteroidetes* and *Proteobacteria,* as well as a significant decrease of *Firmicutes* in the active microbiota, based on 16S rRNA transcripts compared to the general gastric aspirate microbiota according to DNA isolation (Figure 15).

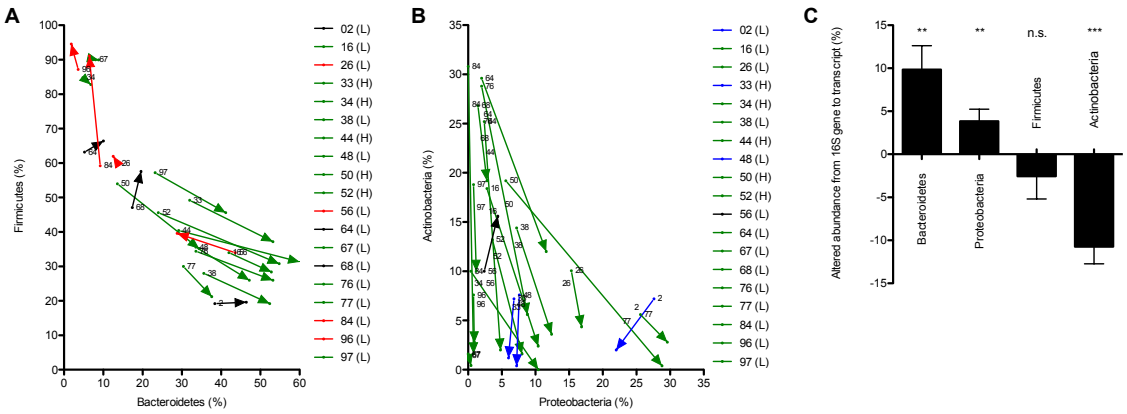


Figure 15. Metabolically active gastric aspirate microbiota. Shifts in the relative abundance when comparing taxonomic microbiota compositions based on 16S rRNA analysis of DNA (arrow base) and RNA (arrow tip) isolates from the same sample are shown for *Firmicutes* and *Bacteroidetes* (A) and *Actinobacteria* and *Proteobacteria* (B). (C) Percentage deviations in relative abundance between phyla concentrations in transcripts and genes. Significant differences were determined based on one sample t-test against a theoretical mean of 0.0 (**p<0.01, ***p<0.001). Numerical data from Rosenvinge et al (138) was reanalyzed for this work. The sample IDs in (A) an (B) refer to the original publication. The data was graciously provided by corresponding author Prof. W. Florian Fricke.

In summary the data of the gastric aspirate microbiota shows a big variations in relative abundances of *Firmicutes* and *Bacteroidetes*. This variation is stable independent from disease state and throughout different studies. Interestingly the luminal gastric microbiota high in *Firmicutes* is more similar to esophagus microbiota. Additionally the taxonomic composition based on RNA transcripts from metabolic active bacteria shows a significant increase of relative abundance from *Bacteroidetes* and *Proteobacteria*. This points towards a selection for bacteria from the phyla *Bacteroidetes* and *Proteobacteria* in the stomach.

2.3.5. Human gastric microbiota compared to mouse and gerbil

Further we wanted to analyze differences of the gastric microbiota in animal model systems that are relevant for research of gastric entities especially *H. pylori* associated gastritis. Therefore we analyzed whole-stomach samples, including its content, from Mongolian gerbils and C57BL/6 mice and compared their microbiota to human stomach aspirate microbiota. The samples were collected from healthy,

co-housed, adult animals. For data analysis reads were rarefied to a sequencing depth of 1415 reads per sample. Total reads and assigned OTUs at maximum depth are significantly increased in human aspirates and gerbil stomach samples compared to mouse stomach samples. Rarefication to 1415 reads/sample leads to an offset in mean assigned OTUs per sample in each tested category. Therefore we can exclude varying sampling depths as a source for evaluation bias. The rarefied depth is applied on the minimum count per sample used in this dataset (human aspirate, gerbil and mouse stomach samples) according to the aspirate sample from P10 (Figure 16).

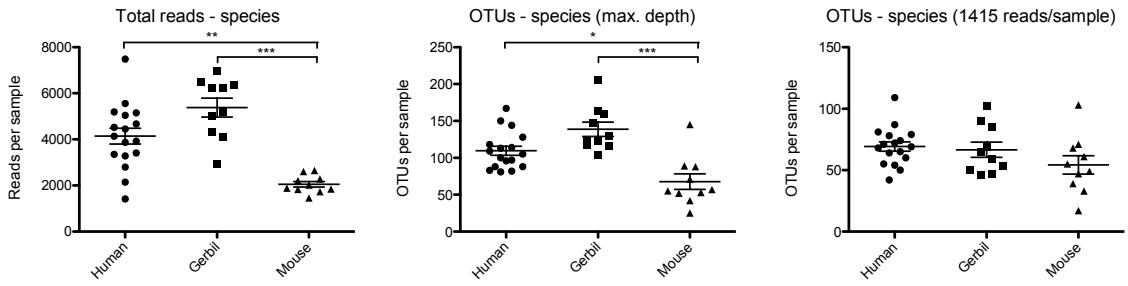


Figure 16. Distribution of generated reads and OTUs used for data analysis in human gastric aspirate, gerbil and mouse stomach samples. The total reads and OTUs per samples were compared regarding the different stomach content between humans, gerbils and mice. Samples from mouse stomachs have significantly decreased generated reads and OTUs at max. depth compared to those of gerbils and human aspirate. At the applied rarefication depth of 1415 reads per sample, there is no significant difference regarding the total number of OTUs between all three species (Kruskal-Wallis test with Dunn's multiple comparison test; *p<0.05, **p<0.01, ***p<0.001).

PCoA with different phylogenetic and quantitative or qualitative distance metrics (unweighted, weighted UniFrac and Bray Curtis) show that the microbiota of different gastric samples cluster by host and that they are significantly separated from each other (p=0.001, non-parametric ANOSIM test; Figure 17).

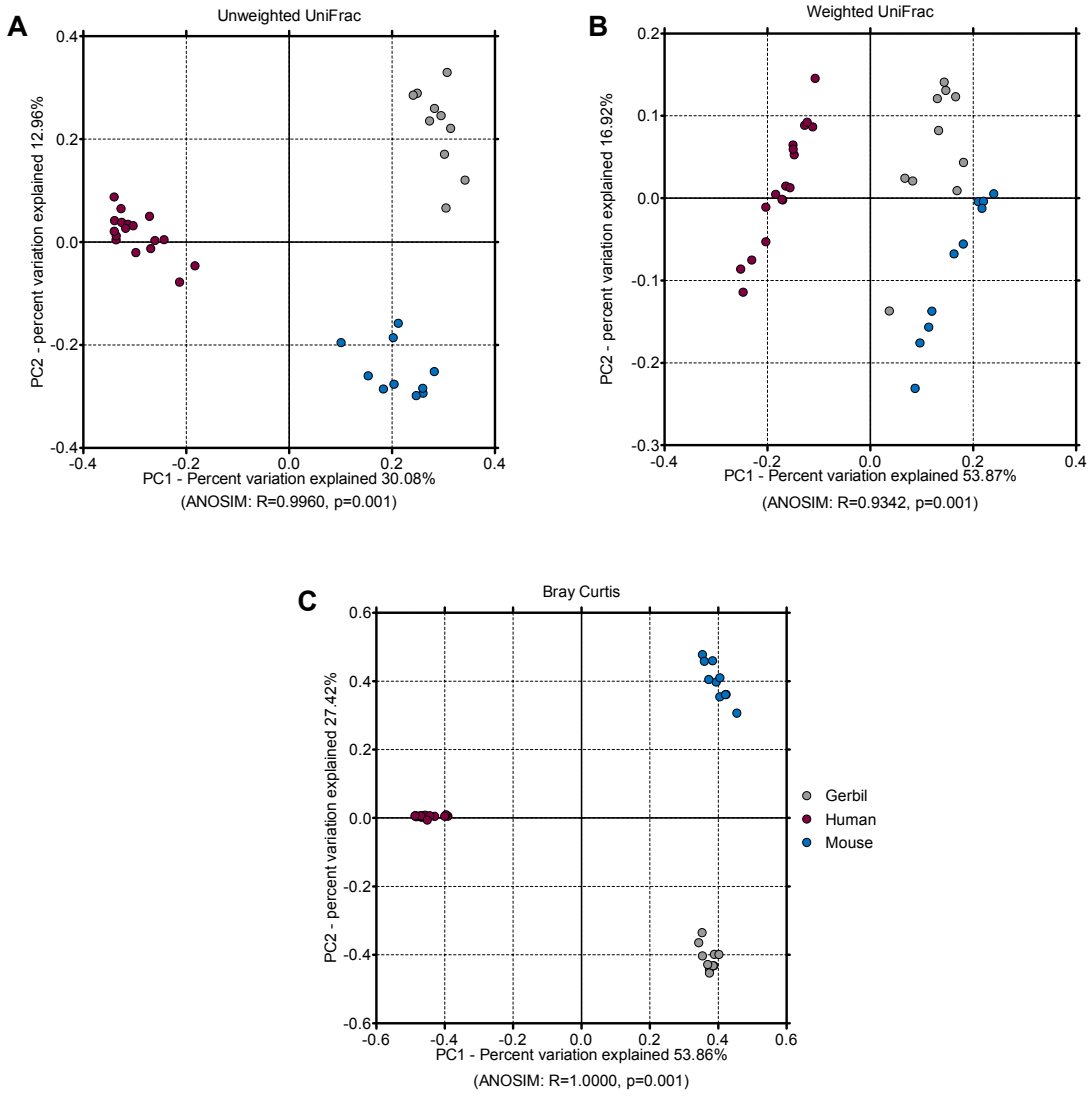


Figure 17. Microbiota analysis of gerbil, mouse and human stomach. Whole-microbiota comparisons based on taxonomic distance (A: unweighted UniFrac; B: weighted UniFrac; C: Bray Curtis) show a significant separation and cluster according to species, as determined with non-parametric ANOSIM test, using 999 permutations.

Additionally we were able to identify a significant increase of microbial diversity in human aspirates compared to gerbil and mouse stomach samples based on the Simpsons index at a rarefication depth of 1415 reads per sample (mouse vs human, gerbil vs human: p= 0.003). Also the Shannon index shows a significant increase as a measure of diversity. This parameter incorporates relative abundance and evenness (mouse vs human: p=0.003; gerbil vs human: p=0.006; Figure 18).

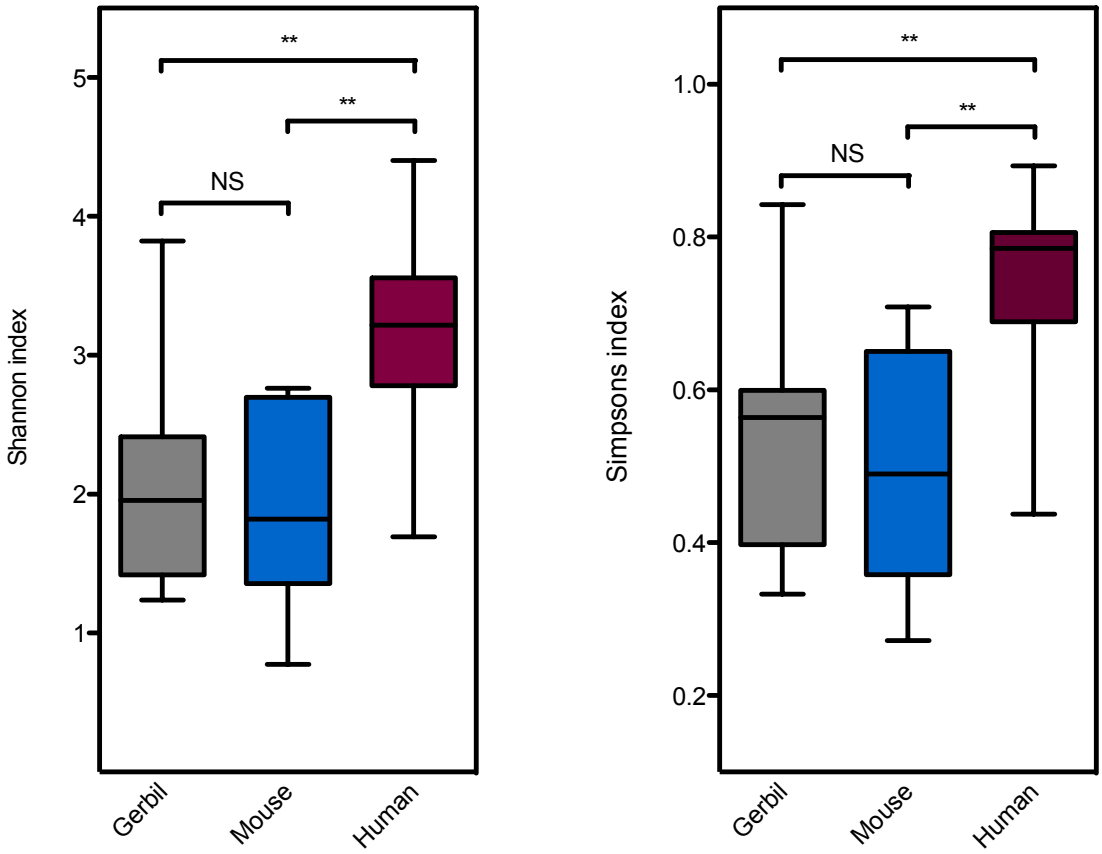


Figure 18. Microbiota comparison of human gastric aspirate with mouse and gerbil stomach samples. Microbial diversity comparison using Shannon and Simpson indices with significance determined based on a non-parametric two sample t-test, using 999 Monte Carlo permutations and Bonferroni correction for multiple comparisons (**p<0.01).

The taxonomic composition shows that the same bacterial phyla, classes and orders, *Firmicutes* / *Bacilli* / *Lactobacillales* and *Bacteroidetes* / *Bacteroidia* / *Bacteroidales,* are dominating the gastric microbiota of human, gerbil and mouse samples. Interestingly on genus level there is a clear difference between human and animal samples. Whereas the dominant taxa within *Lactobacillales* and *Bacteroidales* in human aspirate samples belong to genera *Streptococcus* (59.5 +/- 13.5%) or *Prevotella* (12.9 +/- 2.2%), respectively, gerbil and mouse stomach samples were dominated by *Lactobacillus* (81.9 +/- 13.8%) and an unidentified taxon form the uncultured family S24-7 (8.5 +/- 13.8%) (Figure 19).

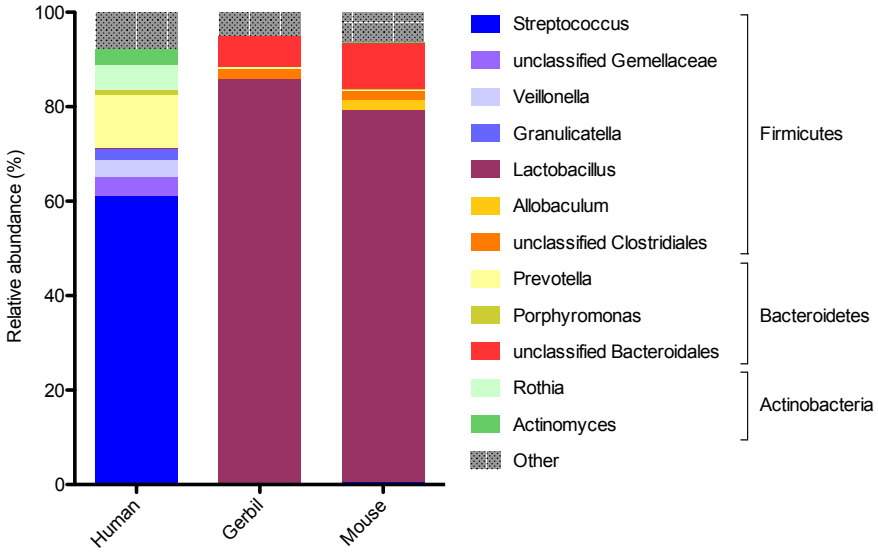


Figure 19. Taxonomic composition of human stomach aspirate, gerbil and mouse stomach samples. Mean relative microbiota compositions at the taxonomic genus and phylum levels, including all OTUs with ≥1% relative abundance in at least one group of samples (human, mouse or gerbil).

Gerbil and mouse stomach samples showed similar variations in the relative abundance of *Firmicutes* and *Bacteroidetes* as human gastric aspirate samples. *Firmicutes* range from 65.3-99.0% in gerbils and from 73.4-99.5% in mice. *Bacteroidetes* vary in relative abundance from 0.6-28.7% in gerbils and from 0.3-26.4% in mice (Figure 20).

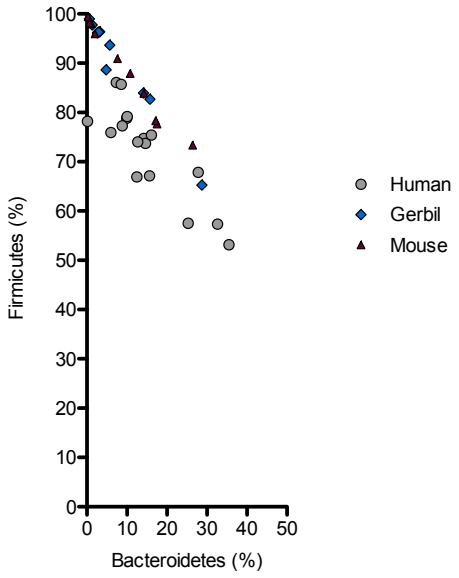


Figure 20. Variation in the relative abundance of *Firmicutes* and *Bacteroidetes* among human aspirate, mouse and gerbil.

Taken together this data show that the gastric microbial communities are unique for their host. The human gastric aspirates microbiota shows an enriched diversity, and is dominated by *Streptococcus* while rodents are dominated by *Lactobacillus*. Despite these strong differences all hosts show similar variations in relative abundances of *Firmicutes* and *Bacteroidetes* in the gastric microbiota.

2.4. Discussion

This study analyzed samples from the upper GI tract from 17 different patients as well as 10 gerbils and 10 mice, which represent important model organisms for gastric cancer and microbiota research. The findings add support to a model of a continuous and dynamic microbial ecosystem of the GI tract. Similar results have been shown in recent microbiota studies which showed for instance the presence of oral taxa in stomach samples (144,145). Bassis et al. even exemplified an overlap of oral and nasal microbial communities with the stomach microbiota (146). A different study was able to identify eight abundant species from the oral microbiota within the fecal microbiota of the same individuals on DNA and RNA level (e.g. *Streptococcus salivarius* and *Haemophilus parainfluenzae*). The abundance level suggest that certain species from the oral cavity survive the transit through the gut but do not represent a dominant part of the microbial community in the lower GI tract (147).

Despite the variations of epithelial organization and associated pathologies of different stomach locations (148), there were only small differences in the composition of the mucosal microbiota between cardia, corpus and antrum. Compared to the microbiota of the esophagus, the stomach microbiota showed a different taxonomic composition. While the relative abundance of *Streptococcus* spp. was increased in esophagus, the relative abundance of *Rothia mucilaginosa, Porphyromonas* and *Lachnospiraceae* were increased in stomach samples. Additionally the concentrations of these taxa significantly correlated with the sequence of different locations downstream the upper GI tract (esophagus > cardia > corpus > antrum > duodenum). One reason for the differences in the microbial composition could be the diverse histological architecture of the mucosa.

Whereas the mucosa of the esophagus is a stratified squamous epithelium consisting of multiple layers of squamous cells, the stomach mucosa is a single-layered epithelium of columnar cells and various mucus secreting cells (148). Decreasing and increasing relative abundances of certain bacterial groups from esophagus to duodenum might indicate that the stomach serves as a bottleneck for bacterial transfer from upper to lower GI tract, although there were no significant taxonomic differences between mucosal microbiota from stomach and esophagus. A study that compared gastric and duodenal aspirates from healthy individuals showed that the relative abundance of *Porphyromonas* and *Lachnospiraceae* is decreased and the concentrations of *Streptococcus* is increased in duodenal aspirates samples (149). Results shown in Figure 15 support these findings as the mean relative abundance of *Streptococcus* increases and of *Porphyromonas* as well as *Lachnospiraceae* decreases in duodenum mucosa microbiota compared to stomach mucosa microbiota. These findings indicate an important role of the stomach whether (ingested) microbes can access the lower GI tract. Thereby the stomach potentially reduces the risk of infections with pathogens, such as *Clostridium difficile* infections, where an association could be shown for a higher risk of *C. difficile* infection in patients receiving proton pump inhibitor (PPI) therapy (150). Also the risk of infections with other pathogens such as *Salmonella* spp., *Shigella* spp., *Campylobacter* spp. and other enteric infections is increased during therapy with acid blockers (151,152). The increased risk of enteropathogen infection is likely be influenced by the patients gastric microbiome, as profound changes of the gastric microbiome in general could be shown after therapy with PPIs and the subsequent decrease of stomach acid production (153). Therefore the acidic changes from gastric to duodenal environment may also affect the mucosal microbiota. The median intraluminal pH shifts in fasting healthy subjects in stomach from 2.05 to 4.90 in the duodenum (154). It could already be shown that different levels of gastric pH significantly changes the phylogenetic diversity of the gastric microbiota (138). This study is therefore also relevant to shed light on the commonly used but poorly understood term of GI dysbiosis (155).

A significant correlation comparing the taxonomic distance between esophagus mucosa and gastric aspirates, and the concentration of *Streptococcus* spp. in the

gastric aspirate respectively, showed that the similarity between esophagus and gastric aspirate microbiota, strongly depends on the relative abundance of *Streptococcus* spp. in gastric aspirates. Although we did not investigate longitudinal sampling to confirm intra-individual temporal variations, the differences in the concentration of *Streptococcus* spp. could dependent on temporal fluctuations of ingested oropharyngeal microbes. Based on the limitations of 16S rRNA amplicon microbiota analysis, which only provides relative abundances and does not provide any absolute values to quantify bacterial loads, the varying *Firmicutes* and *Bacteroidetes* concentrations in the stomach could result from concentration shifts of either one of both dominant groups in the microbiota. However our data indicates that the *Firmicutes* dominated gastric fluid microbiota is more similar to the microbiota of the esophagus, hence the variations of *FIrmicutes* and *Bacteroidetes* ratios could originate from a more constant background of remaining *Bacteroidetes* in the stomach.

This study compared gastric microbiota compositions of three different hosts: Humans, across two heterogeneous patient populations, mice and gerbils. Human and animal samples are dominated by the same two orders *Lactobacilllales* and *Bacteroidetes*. But while human gastric microbiota is dominated by the genera *Streptococcus* (*Lactobacillales*) and *Prevotella* (*Bacteroidetes*), animal microbiota is primarily dominated by the genera *Lactobacillus* (*Lactobacillales*) and an unidentified genus from the bacterial family S24-7, that was recently defined as "*Candidatus Homeothermaceae*" (156), (*Bacteroidetes*). These different microbial patterns on lower taxonomic ranks could play a crucial role in the observed differing colonization resistance and pathology of gerbils and mice infected with human isolates of *H. pylori* (157). Independent from the host, the variations in relative abundance of both dominant bacterial taxa showed a similar distribution. These variations were not associated with health status, sex age or other environmental factors. Therefore we suggest that the intra-individual fluctuations are caused by as of yet unknown non-health-related factors. Lots of studies are correlating certain microbiota profiles with specific medical conditions simply based on the relative abundance ratios between *Firmicutes* and *Bacteroidetes,* e.g. *in human obesity* (102). However studies have shown significant changes in the taxonomic profile, including *Lactobacillales* (*Firmicutes*) and *Bacteroideales*

(*Bacteroidetes*), based on diurnal oscillations independent of health status in human and mice. Thaiss et al. even pointed out that jet lag causes dysbiosis with substantial variations in *Firmicutes* and *Bacteroidetes* before, during and after jet lag (158). Moreover it was reported that the stool consistency strongly influences the composition of the fecal microbiota. The relative abundance of *Bacteroides* (*Bacteroidetes*) positively correlates with looser stool, while *Ruminococcacea* (*Firmicutes*) shows negative correlation. Also a significant taxonomic shift of phyla *Firmicutes* and *Bacteroidetes* can be induced by osmotic diarrhea (159). In addition to our study these findings suggest to be careful with interpretations of relative taxonomic microbiota abundances associated to certain diseases as multiple non-disease related factors are up to be investigated for a detailed understanding of microbial dynamics in the gut.

3. Antibiotic-associated apoptotic enterocolitis in the absence of a defined pathogen: The role of intestinal microbiota depletion.

3.1. Introduction

Antibiotic therapy often results in mild to severe diarrhea and colitis, especially in critically ill patients. Often no specific infectious agent can be discerned (160). It is already known that antibiotic induced depletion of the endogenous microbiota followed by an overgrowth of certain pathogens can cause severe colitis like by *Clostridum difficile* or *Klebsiella oxytoca* (111,161). Aside antibiotic therapy also immunosuppressive drugs can cause diarrhea and inflammation of the GI tract (162,163). A combination of both, an antibiotic and an immunosuppressive therapy might be even more harmful to the intestinal homeostasis than each individual therapy alone. Because both a healthy immune system as well as the gut microbiota have crucial roles in maintaining intestinal homeostasis (164).

This case report of three independent cases, wherein patients represented with antibiotic associated apoptotic enterocolitis during their stay in the intensive care unit (ICU), illustrates potential consequences of a combined antibiotic and steroid treatment in a sterile surrounding. Through immunophenotyping by immunohistochemistry (IHC), microbiota analysis with next generation 16S rRNA gene sequencing and qPCR, as well as determination of bacterial metabolites with gas chromatography - mass spectrometry (GC-MS), we were able to identify several important microbial, epithelial and immune determinants affected in disease.. A single FMT, that was performed in one patient, could counteract dysbiosis of the microbiota and resulted in clinical improvement and healing of the presented disease, highlighting the potential of therapies aiming in restoration of an altered microbiota (1).

Parts of the Results and Figures used are adopted according to the published manuscript: Wurm, Spindelböck et al., Critical Care Medicine 2017 (1).

3.2. Materials and Methods

This methodological description has also been published in an original article (1).

3.2.1. Immunohistochemistry.

Sections of formalin-fixed paraffin-embedded tissue specimens were stained with monoclonal mouse anti-human CD3 (clone F7.2.38; dilution 1:50; DAKO Cytokeratin, Glostrup, Denmark), monoclonal mouse anti-human CD8 (clone C8/144B; dilution 1:30; DAKO Cytokeratin), monoclonal mouse anti-human CD4 (clone 4B12; dilution 1:20; Labvision, Fremont, USA), rabbit anti-human/mouse caspase 3 Active (clone AF835; dilution 1:25; R&D Systems) and FOXP3 (clone 236A/E7; dilution 1:500; Abcam), Pan-ZK (clone MNF116; dilution 1:100; DAKO Cytokeratin), Ki-67 (clone MIB-1, ready-to-use, DAKO OMNIS), CMV (clone CCH2 + DDG9; dilution 1:50; DAKO Cytokeratin), Adenovirus (clone 20/11, dilution 1:50; Chemicon) as recommended by the suppliers (1).

3.2.2. DNA extraction & 16S rRNA gene PCR.

Stool samples and mucosal biopsies obtained at endoscopy were immediately frozen and stored at -20°C. DNA extraction from stool samples and biopsies was performed by mechanical lysis with a MagnaLyser Instrument (Roche Diagnostics, Mannheim, Germany) and subsequent total bacterial genomic DNA isolation with the MagNA Pure LC DNA Isolation Kit III (bacteria, fungi) in a MagNA Pure LC 2.0 Instrument (Roche Diagnostics) according to the manufacturer's instructions. For amplification of the bacterial 16S rRNA gene FLX one-way fusion primer (Lib-L kit, Primer A, Primer B; Roche 454 Life Science, Branford, CT) with the template-specific sequence F27 (5'-AGAGTTTGATCCTGGCTCAG-3') and R357 (5'-CTGCTGCCTYCCGTA-3') (139) targeting the hypervariable regions V1 and V2 were used. PCR reactions for each sample were performed in triplicates. Subsequently the amplicons were purified according to standard procedures, quantified, pooled and sequenced with the GS FLX Titanium Sequencing Kit XLR70 (Roche 454 Life Science, Branford, CT, USA) according to manufacturer's instructions. 16S rRNA gene pyro-sequencing data has been deposited in EBI-SRA under the accession number ERP013256 (1).

3.2.3. Microbiota analysis

Raw files from 454 FLX pyro-sequencing were processed with mothur v. 1.31.2 according to the standard 454 SOP of mothur (54). Sequencing errors were reduced using mothur's implementation of PyroNoise (165), and the command pre.cluster (166) was used to remove sequences that arose due to pyro-sequencing errors. Chimeras were removed with UCHIME (57) and non-bacterial contaminants as chloroplasts and mitochondria were removed using the RDP reference (58). The high quality reads were aligned to the SILVA database (56,167). For an OTU based analysis, the processed fasta files from mothur were introduced into QIIME version 1.7.0 (60). Command pick_de_novo_otus.py was used for OTU picking, OTU were formed by clustering with UCLUST (similarity score of 97%) and taxonomy was assigned using the RDP-classifier and Greengenes reference. The biom file was further analyzed with the command core_diversity.py. For this analysis the samples were rarefied to 750 sequences/sample. For single OTUs a standard NCBI nucleotide BLAST with the "16S ribosomal RNA sequences (Bacteria and Archaea)" database was performed. P-values below 0.05 were considered statistically significant ($p<0.05$=*; $p<0.01$=**; $p<0.001$=***). Presented values are always mean ± SEM if not indicated otherwise (1).

3.2.4. qPCR analysis of butyrate producing genes and bacterial load

Quantitative analysis of microbial butyrate kinase (buk) and butyryl-CoA:acetate CoA-transferase (but) genes in stool samples was performed according to Vital et al. (168). qPCR was performed with the Power SYBR® Green Mix (Applied Biosystems) on a LightCycler® 480 instrument (Roche Life Science) according to the suppliers recommendations; the following oligonucleotide primers were used: buk - G_buk_F/R (*Clostridium acetobutylicum*, *C. butyricum*, *C. perfringens*); but - G_Acida_F/R (*Acidaminococcus* sp.), G_Fprsn_F/R (*Fecalibacterium prausnitzii*), G_RosEub_F/G_Ros_R/G_Eub_R (*Roseburia* sp., *Eubacterium rectale*) (168); 16S rDNA - 1132F/1108R (universal) (169).

For the determination of bacterial load in stool samples, DNA was isolated from 250 mg stool samples with the PowerLyzer® PowerSoil® DNA Isolation Kit

(MoBio). The 16S rDNA was quantified in 3µl of the DNA eluate by qPCR (oligonucleotide primers 1132F/1108R; 10) and the signal was correlated to a standard curve of genomic DNA from *E. coli* DH5α with given concentrations. The results were adjusted to 1g of total stool (1).

SCFA measurements by GC-MS. Acetate, propionate and butyrate levels from stool samples were measured by GC-MS using a Thermo Scientific DSQ II™ Series Single Quadrupole GC-MS by electron ionization instrument. 500µl of homogenized stool were mixed with 500µl H3PO4 (0.5%), internal standards (ISTD: 200µl dC2 [500µM] and 200µl dC4 [500µM]) and 600µl methyl tert-butyl ether (MTBE). After centrifugation (10 min; 2.500 rpm; RT) 150µl of the organic phase was used for the measurement. A calibration curve was created with increasing ISTDs mixed with H3PO4 (0.5%) for the analysis. SCFA analysis has been performed by support of Harald Köfeler and Martin Trotzmüller from the Core Facility Mass Spectrometry at Center for Medical research at the Medical University of Graz (1).

3.3. Results

3.3.1. Clinical background of cases

The three presented independent cases exhibit therapy-refractory high-volume diarrhea and show severe symptoms of apoptotic enterocolitis that resembles acute intestinal graft versus host disease (GvHD) (Table 2). All three cases developed this severe form of enterocolitis, not fitting into any established disease entity, after treatment with broad-spectrum antibiotics and steroids (Figure 21). The endogenous GI microbiota in these patients showed a severe reduction in diversity. Therefore an extrinsic restoration of the physiologic GI microbiota by FMT might cause natural recolonization of the GI tract improving the patients' conditions (1).

Table 2. Clinical background of 3 independent cases showing signs of AAA enterocolitis at the ICU.

Case	Gender	Age	Reason for admission to ICU [clinical history]	Onset of diarrhea	Peak stool volume	Progression of disease
A	female	28	Fever of unknown origin, systemic inflammatory response (SIRS)	day 15 on ICU	4.4 L/day	Clinical improvement and relocation to normal ward after 34 days on ICU. Diarrhea subsided after 45 days.
B	male	46	Methotrexate-induced pneumonitis, acute respiratory distress syndrome (ARDS), SIRS [rheumatoid arthritis]	day 25 on ICU	6.0 L/day	Patient died from multiple organ failure (MOF) on ICU day 66.
C	female	16	Multiple trauma incl. severe head injury subsequent to car accident	day 11 on ICU	7.2 L/day	FMT (400mL of fecal solution, donor: patient's mother) 72 days after onset of diarrhea due to ongoing diarrhea without signs of endoscopic or histological improvement. 2 days after FMT significantly decreased stool volume (1 L/day). Full clinical and histological recovery, last follow-up 97 weeks post-FMT.

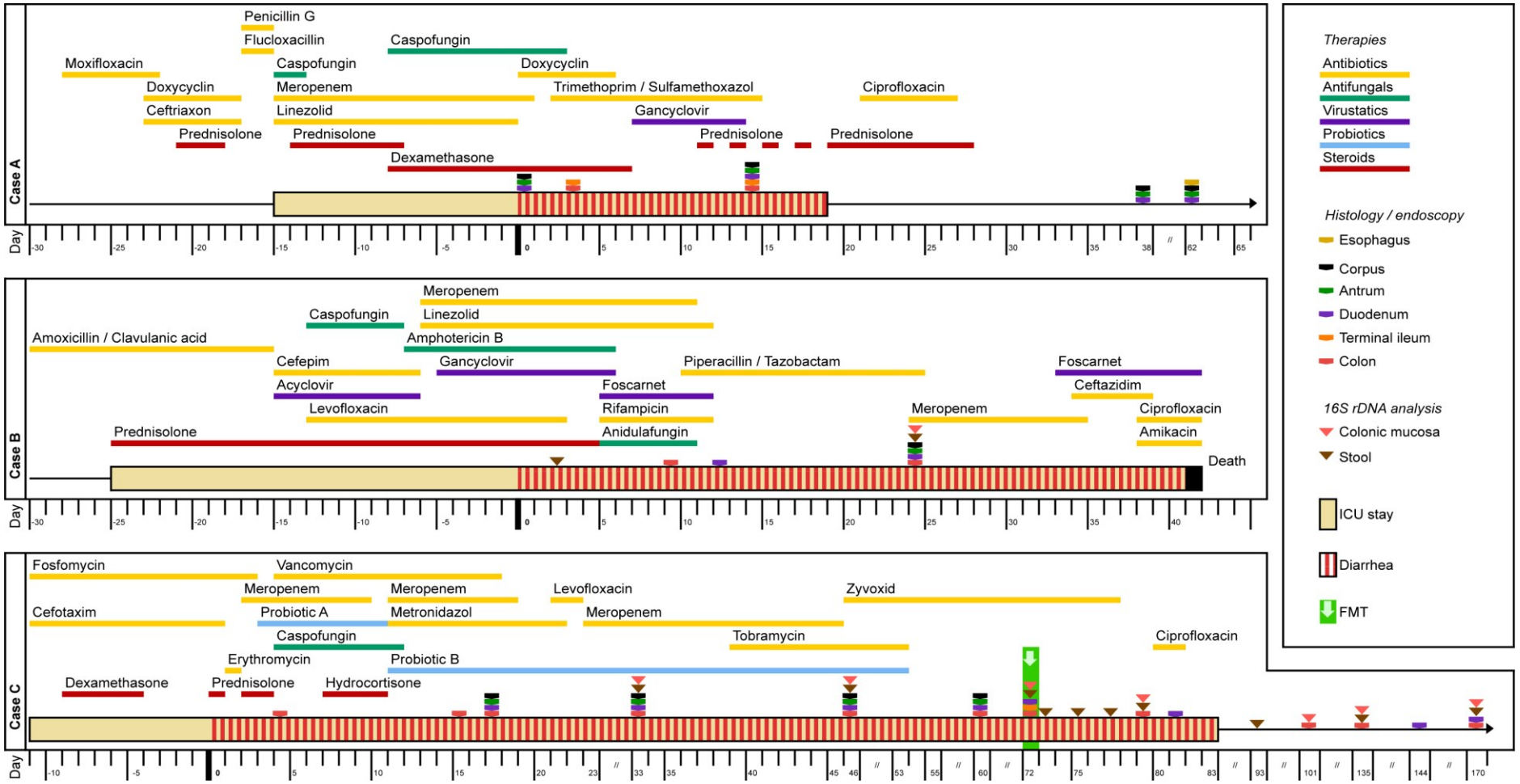


Figure 21. Clinical courses of patients. Admission to the ICU and duration of diarrhea are indicated as boxes at the bottom of each case. Days are counted in relation to diarrhea onset. Therapies including antibiotics, antifungals, virostatics, steroids as well as probiotics are indicated together with endoscopic examinations and samples obtained for histology and microbiota analyses (probiotic A: *Saccharomyces boulardii*; probiotic B: *Lactobacillus acidophilus, L. paracasei, L. rhamnosus, L. salivarius, L. plantarum, Enterococcus faecium, Bifidobacterium bifidum, B. lactis, B. longum*). Adopted from (1).

3.3.2. Endoscopic and Histopathologic Findings in Antibiotic-Associated Apoptotic (AAA) Enterocolitis

In all three cases repeated gastro-duodenoscopies and colonoscopies were performed. They showed severe inflammation in the small and large intestine (Figure 22A). There was segmental loss of folds evident as well as extensive erosions in the duodenum and terminal ileum of all patients (Figure 22B, Figure 23). Additionally manifestations in the colon varied from mosaic like inflammatory changes to large ulcerations and areas with a completely denuded mucosa. Similar to GvHD, a histopathologic hallmark feature in AAA enterocolitis was epithelial mass apoptosis (Figure 22C, Figure 23). In the small intestine this resulted in (sub)total crypt loss and ranged from moderate to severe segmental villus blunting (data not shown).

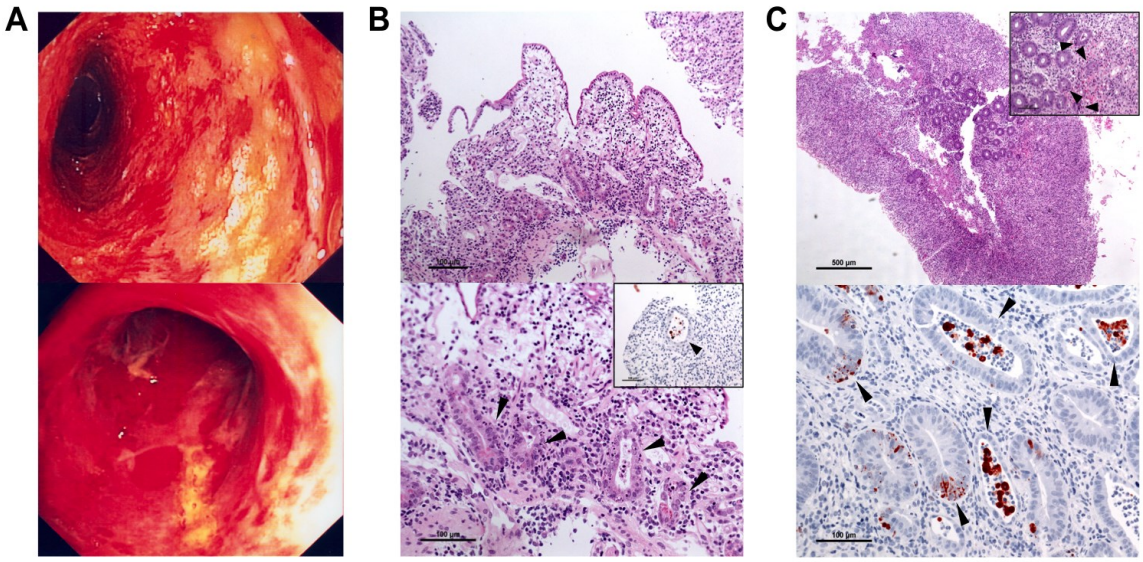


Figure 22. Endoscopy and histology in AAA enterocolitis. (A) Hemorrhagic inflammation with loss of folds in the duodenum (top). Complete loss of vascularization, “geographic” ulcerations with abundant fibrin and denudation in the colon (bottom). (B) Segmental loss of crypts and villi and abundant apoptosis (arrows) in the duodenal mucosa (inset: activated caspase-3 positivity). (C) Large areas completely devoid of crypts and surface epithelium in the colon (inset: crypt epithelial apoptosis). Activated caspase-3 positive crypt epithelial cells and cell debris (bottom). Images: A (top) and B, case A onset of diarrhea; A (bottom), case C 45 d after diarrhea onset; C (top), case C 15 d after diarrhea onset; C (bottom) and inset C, case C 17 d after onset of diarrhea. Adopted from (1).

In the colon the histopathologic features presented from individual crypt losses to large areas with total loss of epithelium. Despite the epithelial damage in AAA enterocolitis, goblet and Paneth cells were present in all three cases. Additionally no signs of viral infections could be detected and patients' biopsies remained negative assessed by repeated testing for viruses and bacterial enteropathogens (data not shown). In summary the patients macroscopically and histopathologically present serious conditions of a severe enterocolitis (1).

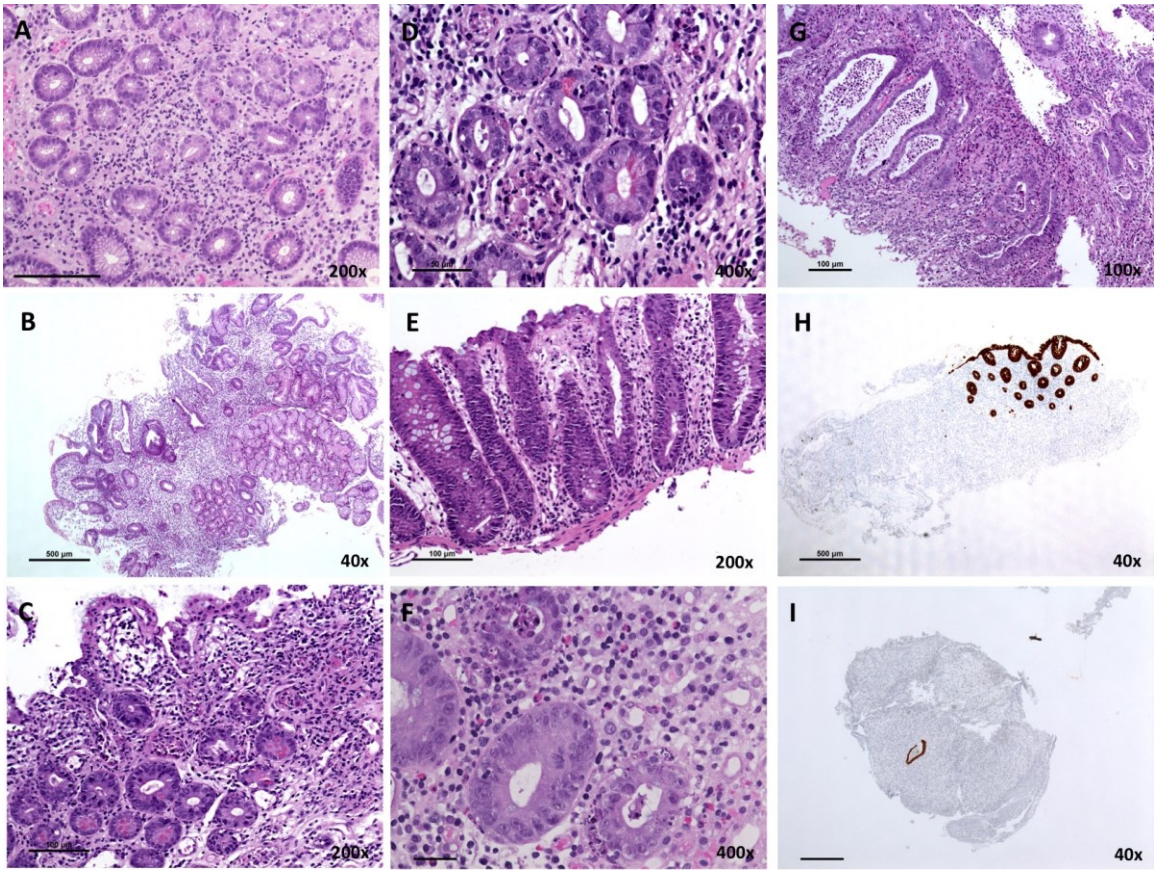


Figure 23. Histological features of AAA enterocolitis. (A) Focally enhanced mild chronic active gastritis (case C, day 33); (B) Duodenal villus blunting and crypt loss (case C, day 17); (C, D) Villus blunting, crypt loss and abundant apoptosis in the ileal mucosa (case A, day 3); (E) Abundant colonic crypt apoptosis (case A, day 3); (F) Abundant colonic crypt apoptosis (case C, day 15); (G) Neutrophilic granulocytes intermixed with cell debris in crypts (case C, day 17); (H, I) Denuded colonic mucosa, remnant crypt epithelium stained with anti-panzytokeratin antibody (case C, day 4 and day 33, respectively). Adopted from (1).

3.3.3. Microbiota Profile in AAA Enterocolitis

Due to the initial clinical findings in the index patient (case A), we subsequently generated longitudinal fecal and mucosal microbiota profiles of case B and case C. The samples that were collected during the acute phase of the disease show a severe depletion of the patient's microbiota. In general a reduction of bacterial load is present in stool samples during the active phase of disease compared to healthy stool samples used for FMT, determined by absolute counts of 16S rRNA gene copies from DNA isolation (Figure 24C). Furthermore the microbiota during the active phase of disease is indicating a severe dysbiosis. In case B 99.9% ± 0.1% and in case C 98.6% ± 1.9% of all OTUs assigned to the phylum of *Proteobacteria* during the active phase of the disease (Figure 24A). Additionally a significant reduction of species richness compared to healthy controls represented by observed species (Figure 24B) and the Simpson reciprocal (or inverse Simpson index) was evident (Figure 25B). 16S rRNA analysis revealed that the patients' microbiota showed strong fluctuation over time during the active phase of the disease as shown in Figure 24A. In case B there were 11 OTUs on day 2, dominated by a single OTU assigned to *Pseudomonas* sp. (98.8%), followed by an evident shift to a microbiota dominated by an unclassified *Enterobacteriaceae* (97.5 ± 0.9%) on day 24. The relative abundance of *Pseudomonas* sp. OTU decreased to 2% ± 0.5% on day 24. The microbiota of case C also showed a low-diversity profile compared to controls dominated by various strains during the course of disease. During probiotic supplementation the patient's microbiota was dominated by strains from the administered probiotics (Figure 21). On day 33 we identified a bloom of *Lactobacillus* sp. (60.4% ± 18.7%) and *Enterococcus* sp. (52.6% ± 9.5%) on day 46. Additionally two strains, that are typical skin commensals, were detectable: *Staphylococcus epidermidis* (34.5% ±10.4%) and *Propionibacterium acnes* (1.8% ± 3.2%). On day 72 after probiotic therapy the microbiota was dominated by *Haemophilus parainfluenzae* (74.2% ± 21.2%). This proteobacterium generally colonizes the oropoharynx and is usually not a colonizing the lower gastrointestinal tract (1). In summary, the dramatic and random fluctuations of taxa and the prevalence of taxa typically colonizing higher (oropharynx) or other habitats (skin) is indicative for a defective intrinsic colonization resistance of the gut's microbial ecosystem (73,170). Antibiotics have

been shown to lower colonization resistance and even create resources for *Enterobacteriacea* by eliminating butyrate producing bacteria. Colonocytes have to switch their metabolism from fermentation of butyrate to fermentation of glucose to lactate. Thereby they are not only increasing the concentration of nitrate (NO_3^-) but also oxygen levels (O_2) in the intestinal lumen. This favours growth of facultative anaerobes over obligate anaerobic bacteria and leads to a bloom of *Enterobacteriaceae* (171).

Clinically Case C's conditions got worse, showing endoscopically persistent inflammation of the left colon and ongoing severe diarrhea (2-7L per day). Considering the patient's severely disturbed and depleted microbiota, FMT was performed trying to reestablish an intrinsic colonization resistance by introduction of a healthy physiologic microbiota, 72 days after onset of diarrhea. The transplant that was used, consisted of 250 different OTUs. 61.7% of all reads were assigned to *Bacteroidetes* and 37.3% to *Firmicutes*. Compared to healthy control stool the transplant is representing a physiologic fecal microbiota (Figure 24D) (1).

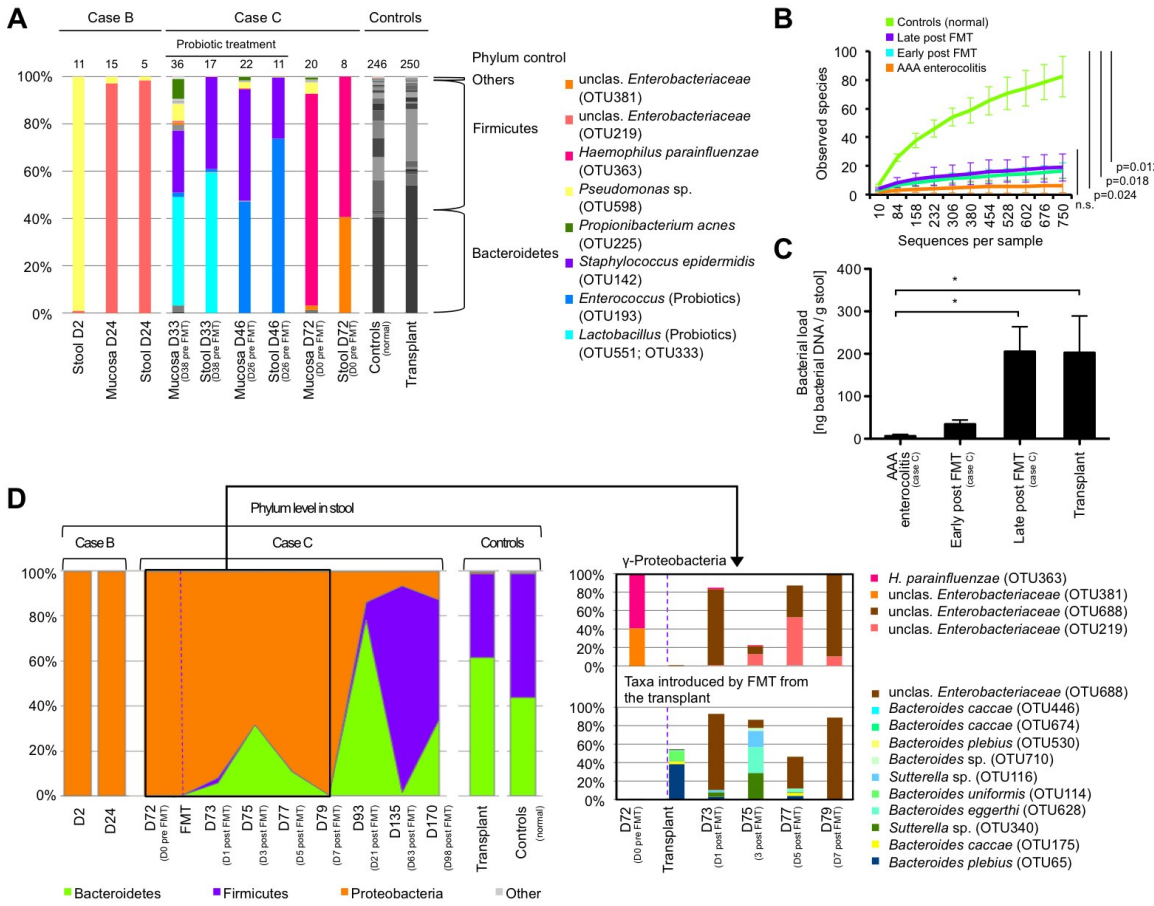


Figure 24. Microbiota depletion and effects of FMT in AAA enterocolitis. (A) Fluctuating taxa during acute disease in cases B and C. A normal colonic microbiota is shown as control (gray bars). The numbers above bars indicate number of prevalent taxa. (B) Microbial richness is impaired in AAA enterocolitis. Neither probiotic treatment nor FMT is able to restore reduced richness (Two-sample t test, 999 Monte- Carlo permutations, Bonferroni-correction, samples were rarefied to 750 sequences per sample). (C) Microbial load is significantly reduced and increases after FMT (one-way analysis of variance [ANOVA], Bonferroni posttest; * $p < 0.05$). (D) Phylum level microbiota dynamics in case C before and after FMT compared with case B and controls (left). Operational taxonomic unit (OTU) level analysis indicates removal of *Haemophilus parainfluenzae* after FMT and recolonization with *Gammaproteobacteria* followed by *Bacteroidetes* originating from the transplant (right). Adopted from (1).

Already 1 day after FMT the relative abundance of *H. parainfluenzae* in the patient's fecal microbiota decreased to 1.5% which was instead dominated by an unclassified *Enterobacteriaceae* (81.5%) that originated from the transplant. Beside this taxon several other strains mainly assigned as *Bacteroides* spp. were successfully introduced into the patients via FMT (Figure 24D). Follow-up samples of the patient show that *Bacteroidetes* and *Firmicutes* strains are able to efficiently recolonize the fecal microbiota over time (Figure 25D). Although there is no

significant increase of species richness in stools post-FMT (up to day 170 after onset of diarrhea), reestablishment of the microbiota was evident by a significant increased bacterial load in stool samples 3 weeks after FMT and later, which reached levels similar to our controls (Figure 24C) (1).

The microbial diversity determined by weighted UniFrac, a quantitative distance metric using phylogenetic distances between observed species, showed that the microbial community is highly similar within all samples during the active phase of the disease, whereas the distance between samples corresponding to an AAA enterocolitis and controls are significantly different. Comparing the distance of patient samples pre and post FMT we see a significant convergence of patient samples towards the donor microbiota in samples taken 3 weeks or later after FMT (Figure 25A, Figure 25C) (1).

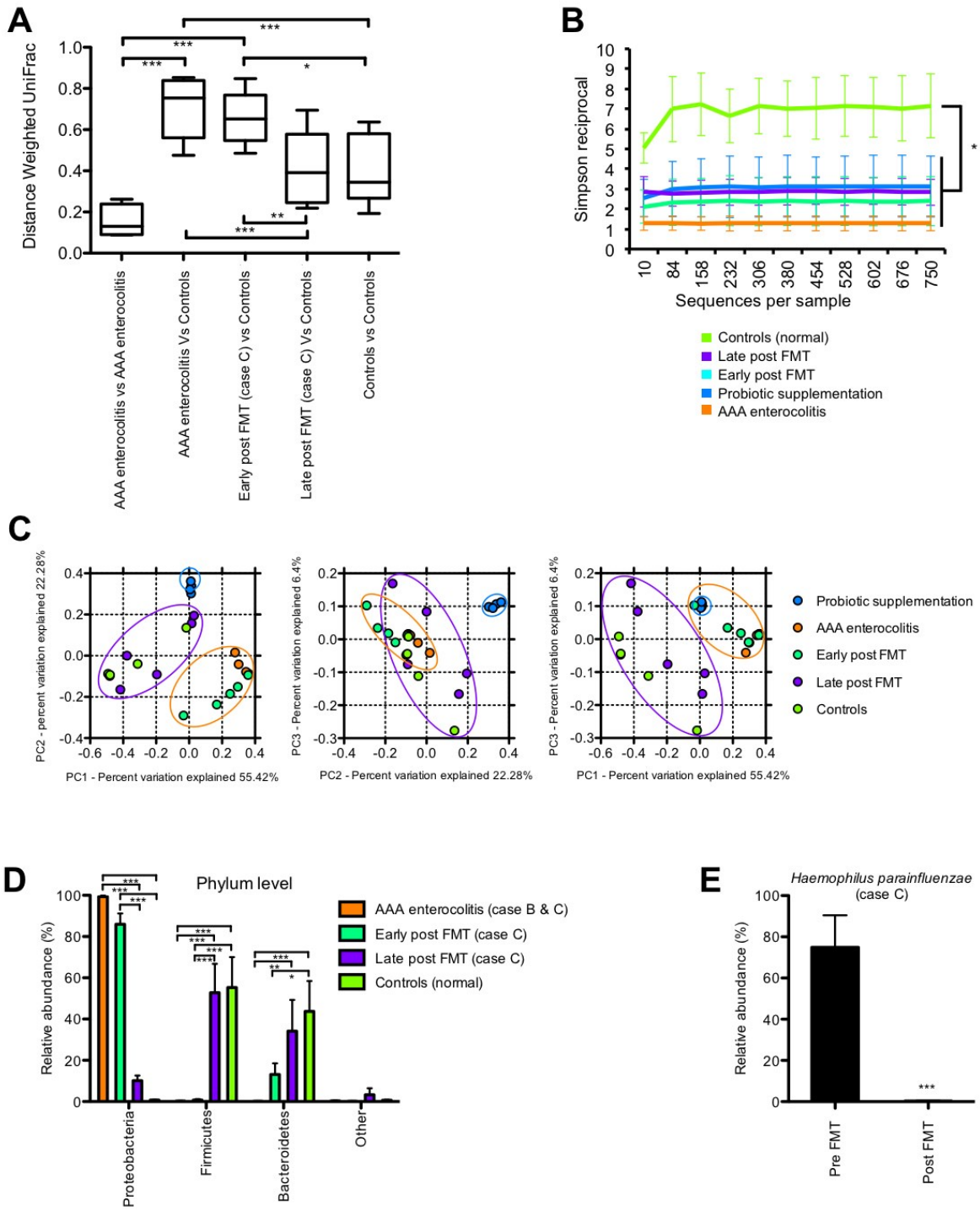


Figure 25. Microbiota dynamics and the effect of FMT in AAA enterocolitis. (A) Comparison of weighted UniFrac distances from stool and colonic mucosa samples of cases B and C at acute phase of disease indicates a more similar microbiota composition of cases when compared to controls. After FMT distances significantly decrease indicating that post transplantation the microbiota of case C approaches towards a normal stool microbiota (note: a low distance value indicates a more similar microbiota composition between assessed samples; two-way ANOVA, Bonferroni post-test; *p<0.05, ** p<0.01, *** p<0.001). (B) Microbiota diversity displayed with the Simpson reciprocal metric. Diversity is highly reduced in AAA enterocolitis and gradually increased during probiotic supplementation and

after FMT, although control samples show a significantly higher diversity compared to all other groups (two-sample t-test using 999 Monte Carlo permutations and Bonferroni correction; $p<0.05$). (C) PCoA plots based on weighted UniFrac distances show a significant grouping of samples originating stool samples from the acute phase of cases B and C together with samples early after FMT from case C. Samples originating from later phases after FMT (latepostFMT: from day 21d post FMT) group together with control samples indicating a reestablishment of a normal microbiota. Samples originating from the probiotic treatment during acute disease of case C group individually (ANOSIM; $p<0.001$). (D) *Proteobacteria* are dominating in AAA enterocolitis and phylum wide microbiota changes due to FMT (sample designations are given in Tab. S4; two-way ANOVA, Bonferroni post-test; *$p<0.05$, ** $p<0.01$, *** $p<0.001$). (E) Opportunistic *H. parainfluenzae* completely diminishes after FMT in mucosa and stool samples of case C (pre FMT: n=2; post FMT n=11; ANOVA, Bonferroni- and FDR-corrected; *** $p<0,001$). Adopted from (1).

3.3.1. Attenuation of Severe Dysbiosis by FMT Was Associated With Resolution of Symptoms and Mucosal Healing in a Patient With AAA Enterocolitis

Associated with regeneration of a physiological fecal microbiota the diarrhea of the patient subsided after day 7. Mucosal improvement was confirmed by histology. As shown in Figure 26A the colonic epithelium shows regenerating crypts and reestablished epithelium lining seven days post FMT (lower panel, left) compared to 12 days pre FMT and on the day the FMT was performed (upper panel, left & right). An antibody staining against Ki-67, a protein that is present in all phases of the cell cycle except the resting phase G_0 and is therefore used as a marker for cellular proliferation, shows active regeneration of crypt epithelium seven days post FMT in case C (lower panel, right). Additionally immunohistochemistry revealed that $CD8^+$ T-cells are significantly increased in colonic mucosa during active phase of the disease, whereas $CD4^+$ T-cells are significantly decreased compared to controls. CD4 is a glycoprotein that is expressed on mature T helper cells, whereas CD8 is expressed on the surface of cytotoxic T-cells. In healthy controls with a balanced immune system this ratio is about 3:1 for CD4 cells whereas the ratio during the acute phase of disease is 1:19 due to infiltration of $CD8^+$ T-cells indicating an imbalanced immune system. This imbalance could be reversed in case C after treatment with FMT (Figure 26B, detailed counts from single biopsies in Figure 27). $CD3^+$ & FoxP3 are markers expressed on regulatory

T-cells (Tregs), which are vital for maintenance of immunological tolerance by ending immune reactions and suppressing autoimmune reactions. We used double staining for CD3 and FoxP3 , but could not identify any significant changes of the Treg population in phase of the disease compared to controls (Figure 26C, detailed counts from single biopsies in Figure 28) (1).

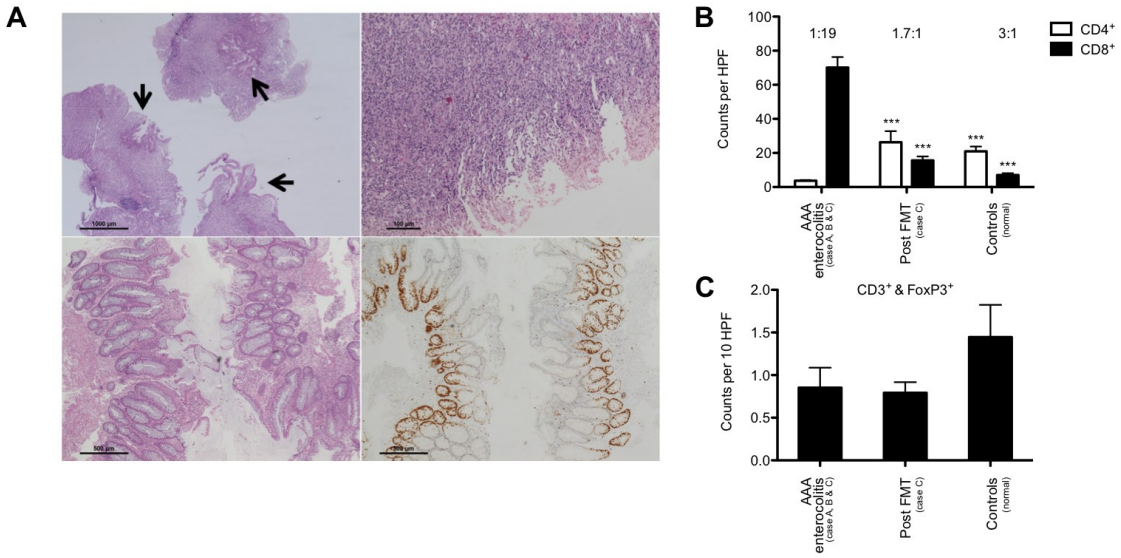


Figure 26. Histopathology and mucosal immunophenotype pre- and post-FMT. (A) Colon histopathology 12 d ahead (top left) and at the day of FMT (top right). Only focal residual epithelium is present (arrows). Seven days after FMT, the epithelial lining is reestablished (bottom left). Ki-67 immunohistochemistry identifies proliferating crypt epithelia (bottom right). (B) Significantly increased CD8+ T cells and significantly reduced CD4+ T cells during acute disease in the colon, this immunophenotype is reversible after FMT (top); CD4-to-CD8 ratio is given above bars (one-way analysis of variance [ANOVA], Bonferroni-corrected, *** $p < 0.0001$). CD3+FoxP3+ double-positive Tregs are not significantly altered (bottom; one-way ANOVA, $p = 0.2801$). AAA = antibiotic-associated apoptotic, HPF = high power fields. Adopted from (1).

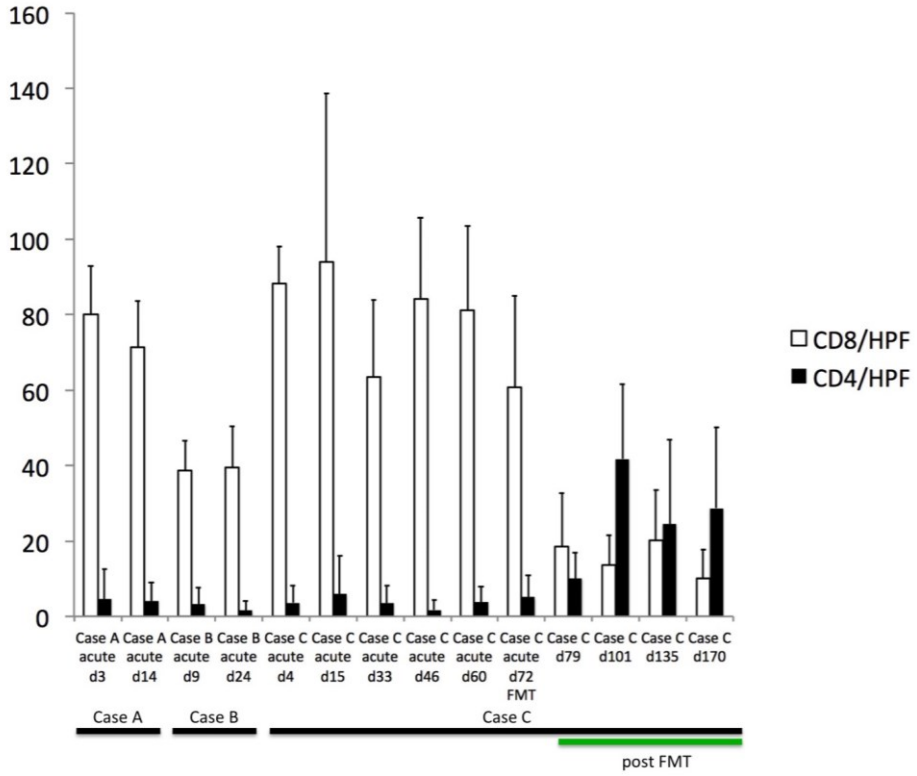


Figure 27. CD8$^+$ and CD4$^+$ T lymphocyte counts in the colonic mucosa of patients with AAA enterocolitis. Assessed by immunohistochemistry (scored per high-power field, HPF). Adopted from (1).

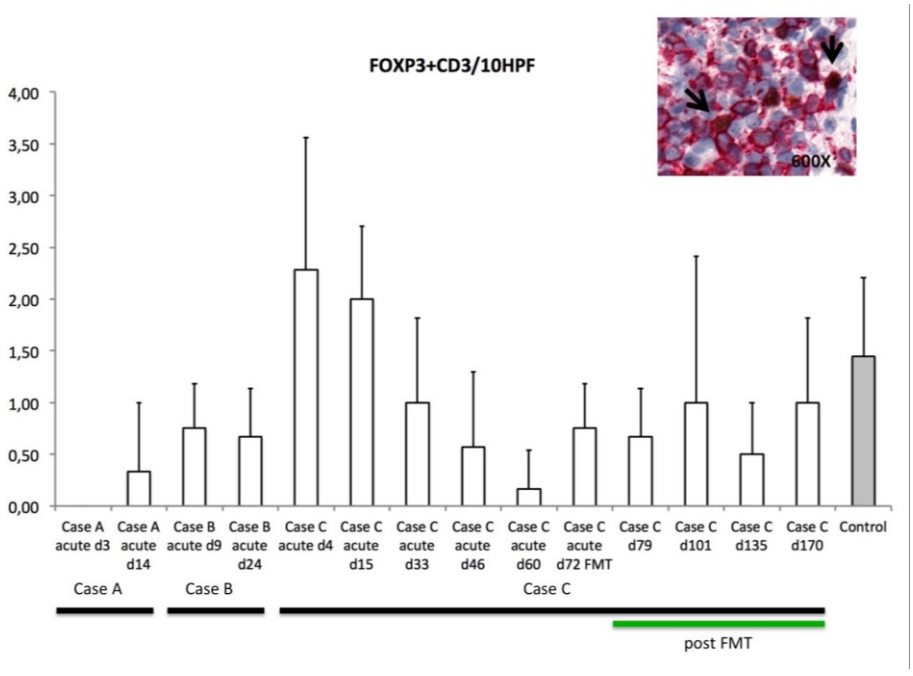


Figure 28. Regulatory T cell counts assessed by double-immunostaining of the markers FOXP3 und CD3 (scored per 10 HPF). Representative picture (inset) of membranous CD3 positivity (red) and nuclear FOXP3 positivity (brown). Double marked cells are indicated with arrows. Days (d) are counted from the onset of diarrhea. Adopted from (1).

It has been shown, that short chain fatty acids (SCFAs), products of bacterial fermentation, positively regulate the homeostasis of colonic T-reg cells (172). Therefore, we performed GC/MS analysis for propionate, acetate and butyrate. Interestingly stools from case C show a reduction of SCFAs during the acute phase of the disease. After the treatment with FMT levels of acetate increased followed by propionate concomitant with an increase of *Bacteroidetes* and *Firmicutes* in the patients stool microbiota. Elevated levels of butyrate were only detectable in the transplanted feces. This could be due to the fact that typical butyrate producers, like *Lachnospiraceae* and *Ruminococcaceae* are not present in the fecal microbiota during the acute phase of the disease and in the early phase after the FMT. In contrast the relative abundance of the transplanted microbiota from *Lachnospiraceae* and *Ruminococcaceae* shows 33% (Figure 29) (1).

To verify the results of the GC/MS analysis, we performed a quantitative PCR for butyryl-CoA: acetate CoA-transferase (*but*) and butyrate kinase (*buk*) genes from the microbial DNA isolated from the stool samples according to Vital et al (168) to see if the genes necessary for production of butyrate are present and if the microbiota has the capacity of butyrate production. The qPCR shows a significant increase of bacterial butyrate synthesis gene *but* in DNA of healthy controls compared to case C (Figure 30). These decrease of *but* genes in DNA isolated from patients fecal samples compared to fecal transplants and normal control stool samples confirms the result of the GC/MS analysis as the patients microbiota lacks the metabolic and genetic capacity to produce butyrate (Figure 29) (1).

Endoscopy performed ninety-seven days after the single FMT showed an almost normal and healthy colonic and duodenal mucosa (Figure 31) (1).

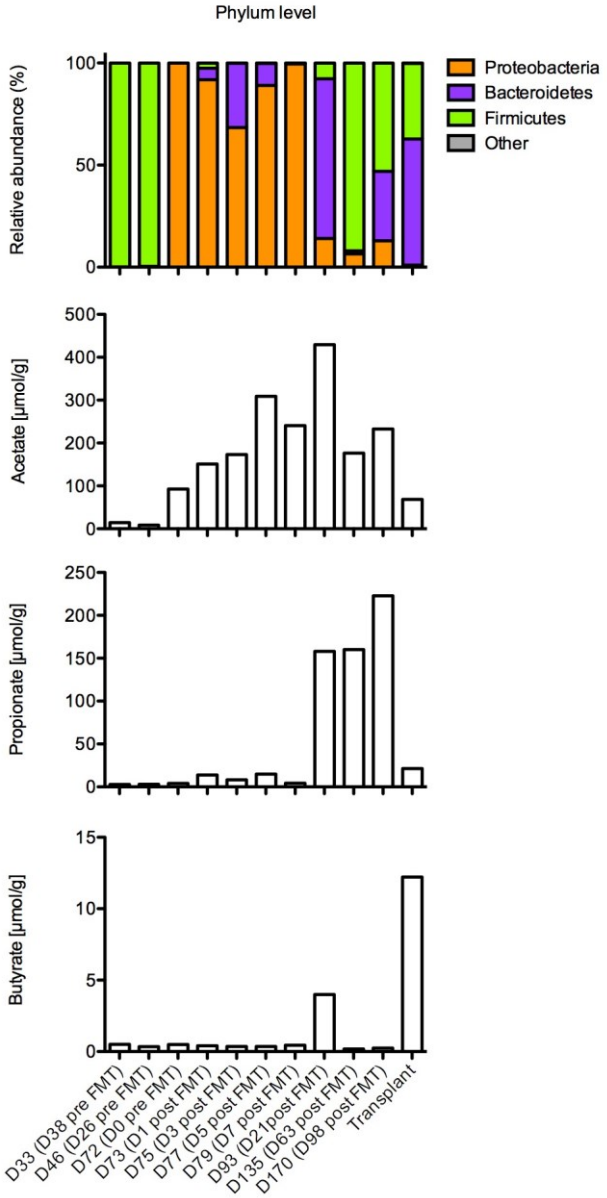


Figure 29. Short-chain-fatty-acid levels in stools of case C. GC-MS analyses of SCFAs in case C pre and post FMT compared to the transplant. SCFAs are broadly decreased during the acute phase. A prominent increase of acetate levels in stools, correlating with the recolonization of *Bacteroidetes*, and an increase of propionate levels during recolonization of *Firmicutes* could be detected directly after FMT. No significant increase of butyrate levels was noted. The lack of butyrate post FMT might be due to low-level colonization of typical butyrate producers like members of the families *Lachnospiraceae* and *Ruminococcaceae* (0-7% abundance); at D93 3.99 µmol/g butyrate was measurable correlating with 7% *Lachnospiraceae* and *Ruminococcaceae*). The transplant shows elevated butyrate levels and a relative abundance of 33% of these well-known butyrate producers. SCFA analysis has been performed by support of Harald Köfeler and Martin Trotzmüller from the Core Facility Mass Spectrometry Graz. Adopted from (1).

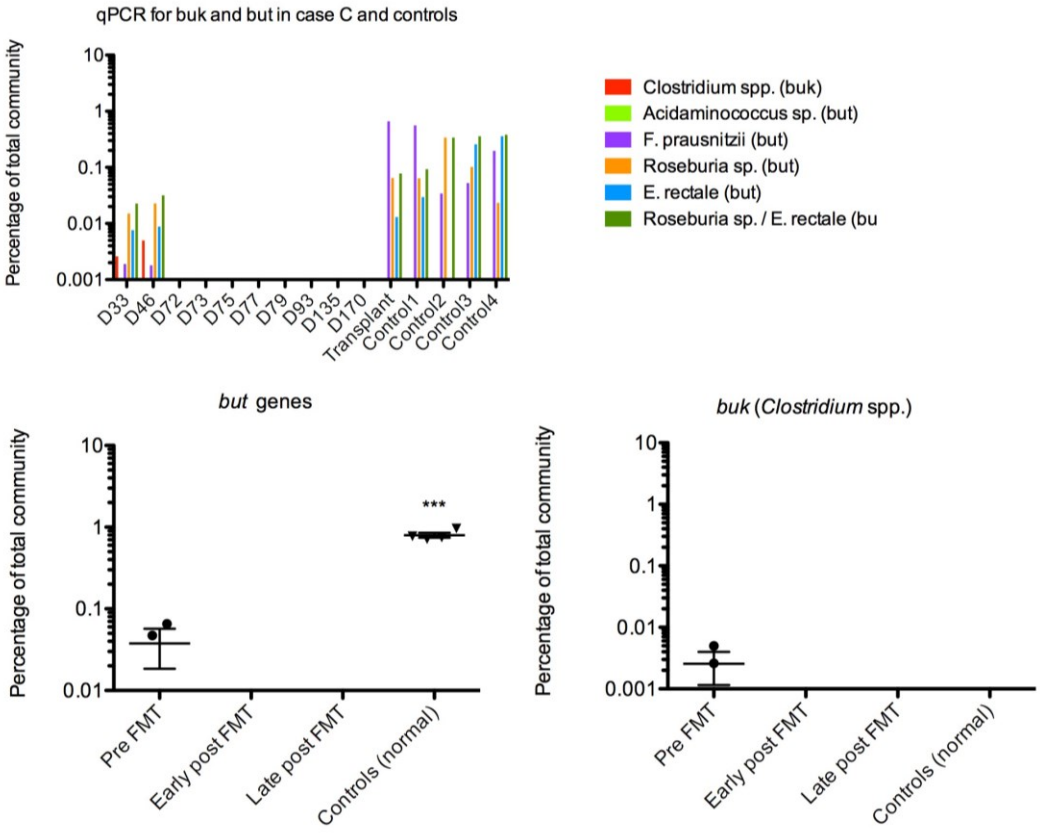


Figure 30. Butyrate producing genes in fecal specimens of case C. qPCR of *but* genes compared to total 16S RNA genes shows a significant increase in control stools compared to stools from case C pre and post FMT (One-way ANOVA, *** $p<0.0001$; note: positive samples for *but* pre FMT were taken during probiotic treatment). No significant difference between groups could be measured for *buk* genes. These results correlate with the GC-MS data showing no increase of butyrate in feces of patient C post FMT. Adopted from (1).

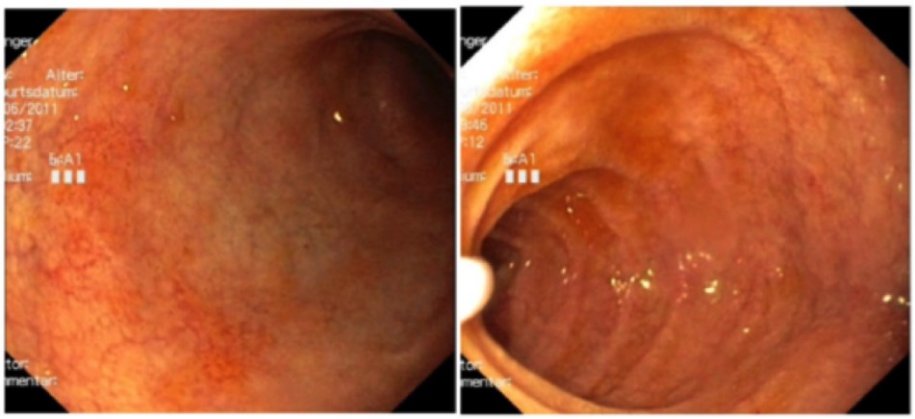


Figure 31. Endoscopic appearance 97 days post FMT in case C. Only a discrete mucosal hyperemia is visible in the duodenal (left) and colonic mucosa (right). Adopted from (1).

3.4. Discussion

This study is based on three independent cases and describes a severe form of AAA enterocolitis in patients that were initially treated at the ICU based on other diseases. All patients shared several common features as they were all treated with a combination of steroids and broad-spectrum antibiotics when intractable high-volume watery diarrhea and GI failure suddenly emerged. They display a severe apoptotic enterocolitis in histopathology, which resembles acute GvHD despite the absence of any hematopoetic or solid organ transplantation. There was also no detectable enteropathogen in cases. The course of disease was different in each patient. Whereas one patient showed spontaneous recovery after being discharged from ICU after 2 months, one patient died in the ICU 6 weeks after onset of diarrhea due to multiple organ failure (MOF). The third patient showed improvement after being treated with a single-FMT. As already reported elsewhere changes of the gut microbiota can be a consequence of critical illness which can already be caused by severe insult or trauma alone (173–175). We suggest that the prolonged antibiotic therapy and concomitant microbiota depletion is a contributing factor to trigger inflammation, facilitating overgrowth of opportunistic pathogens, and resulting in a lack of anti-inflammatory bacterial metabolites, such as butyrate and other short chain fatty acids (176). These findings clearly indicate new, alternative disease models for antibiotic-associated colitis. Important to note, although no classical enteropathogens were detected in cases, other unidentified pathogens, like viruses and fungi could be possibly involved. Despite the dramatic improvement of disease after FMT, also other factors such as the withdrawal of corticosteroids, changes in antibiotic treatment or continued supportive care could have beneficial effects on the healing process in patient C. Noteworthy, FMT is used as therapy for refractory *C. difficile* infection (CDI) (107,177,178), and has been used for severe or life-threatening forms of CDI (179–182). Thus it seems to be a safe procedure. Despite the transfer of billions of unknown microorganism into an inflamed gastro-intestinal tract, the events of short-term infections or gut-derived septicemia are quite rare (183,184). While application of FMT into the upper GI tract through a nasojejunal tube can lead to aspiration of fecal material and consequently to severe pneumonia or death (185,186), the administration of FMT into the lower gastro-intestinal tract via

colonoscopy seems to be tolerated very well showing barely side effects (183,187). Additionally a study of FMT treatment for CDI in immunocompromised patients showed that even patients with an attenuated immune system only show few side effects to this treatment (188). Nevertheless there are still limited reports giving indication on side effects, the risk of viral transmission or translocation of microorganisms with consequent septicemia. Furthermore the transfer of various disease-related phenotypes that are associated with a certain type of microbiota cannot easily be predicted (184,189). Therefore FMT as a treatment of choice for non-CDI indications still entails unforeseen risks. To reduce these risks further studies need to be carried out and donor selection have to be performed carefully granting certain health criteria (1).

In summary this report identifies AAA enterocolitis in three independent critically ill patients. All patients developed a severe form of enterocolitis characterized by mass-apoptosis of the gastro-intestinal epithelium, resembling GvHD, after receiving a concomitant antibiotic-corticosteroid therapy. Additionally they suffered from a high-volume diarrhea accompanied with strong fluctuations and changes in the taxonomic composition of the gastro-intestinal microbiota. The dysbiosis of the fecal and colonic microbiota was determined by a loss of bacterial load in the fecal matter and a few dominating taxa based on their relative abundance 16S rRNA gene copies per sample. Especially in patient C longitudinal samples show that the dominant taxa are strongly fluctuating within the microbiota. The fact that the identified taxa were assigned to known 16S sequences of either administered probiotic strains or skin and oroharynx microbes, that generally do not colonize the human gastrointestinal tract, indicates a strong bacterial transition of microbes acquired from any superior source. Therefore we hypothesize that the altered mucosal and fecal microbiota in this patients could result from absence of intestinal colonization resistance. This absence might be restored by the introduction of natural fecal and colonic microbes as a single-FMT treatment was followed by reestablishment of a normal physiologic microbiota. 16S rRNA amplicon sequencing and SCFA analysis by GC-MS display a two-stage process that might be involved in the microbial restoration. In the early-phase post-FMT various *Bacteroides* spp. together with a transplant specific unclassified *Enterococcaceae* were introduced into the intestinal microbiota. For *Bacteroides*

spp. it could already be shown that SCFA and polysaccharide A (PSA) production contribute to epithelial- and immune-homeostasis in the colon mucosa by inducing an anti-inflammatory immune response (172,190). Additionally an increase of acetate could be determined directly after the FMT in fecal samples of the patient by GC-MS. Acetate is the most abundant SCFA in the colon and it is known that *Bacteroides* spp. are able to metabolize pyruvate to acetate (191,192) which can be utilized by the majority of butyrate producing bacteria in the gut via the butyryl-CoA:acetate CoA-transferase pathway (193). After *Bacteroides* spp. recolonization we observed an increase of propionate and butyrate concentration in the later phase post FMT treatment together with an increase of taxa that are assigned to the phylum *Firmicutes.* This observation might be related to the findings of Wrzosek et al. The authors of this study showed that colonization of germ free rats with *Faecalibacterium prausnitzii* (*Firmicutes*) only works if *Bacteroidetes thetaiotamicron* was previously introduced (194). In analogy to these findings, microbiota analysis of fecal samples showed that, although *Firmicutes* (*Clostridia*) were highly abundant and diverse in the transplant (OTUs assigned to *Firmicutes*/total OTUs: 128/181), they were not able to initially colonize after FMT. Almost none of the OTUs assigned to phylum *Firmicutes* post-FMT originated from the transplant. Additionally Wrzosek et al. showed in co-culture experiments, that *F. prausnitzii* is able to convert acetate, produced by *B. thetaiotamicron,* to butyrate. *F. prausnitzii and B. thetaiotamicron* are metabolically complementary and contribute to the establishment of epithelial homeostasis by induction of mucin production and the development of goblet cells in the colonic epithelium of gnotobiotic rats (194). It was also shown that butyrate produced by commensal *Clostridia* (*Firmicutes*) induces the differentiation of colonic Tregs and ameliorates T-cell dependent colitis in *Rag1*$^{-/-}$ mice (87). Both studies support the hypothesis that sequential reconstitution of the physiological microbiota could have been associated with the regeneration of the colonic epithelium in patient C. A stratified process with early colonizers (*Bacteroidetes*) followed by late colonizers (*Firmicutes*) is potentially also needed in intensive patients suffering from microbiota depletion to reestablish the colonic microbiota (Figure 32). It might be that such a stratified reestablishment of the colonic microbiota does not happen spontaneously in the ICU, but is potentially achieved by FMT and thereby leads to a break in the overwhelming inflammation (107,122,195). To strengthen this

hypothesis further workup of microbiota profiles are needed using longitudinal samples from patients in the ICU suffering from a depletion of the intestinal microbiota with subsequent reconstitution by FMT (1).

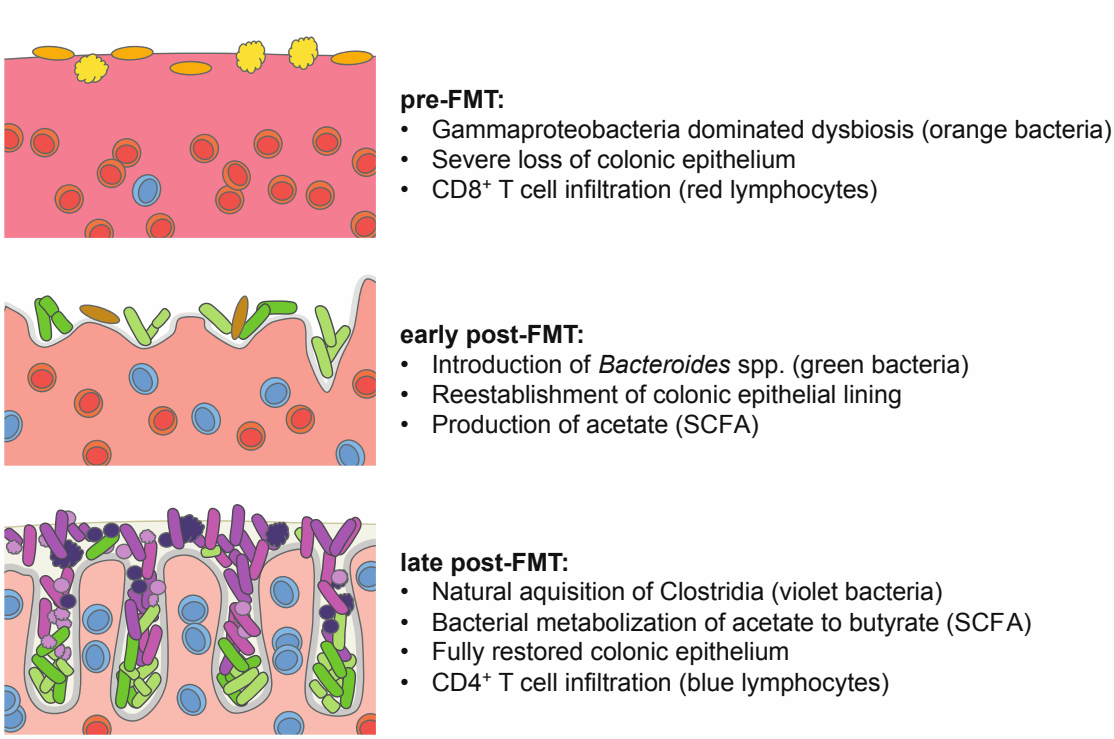


Figure 32. Colonic mucosa and concomitant microbiota: Sequential reconstitution of colonic mucosa in antibiotic associated enterocolitis colitis before and after FMT presenting the regeneration of epithelium and crypts.

4. The taxonomic composition of the donor intestinal microbiota is a major factor influencing the efficacy of fecal microbiota transplantation in therapy refractory ulcerative colitis

4.1. Introduction

Inflammatory bowel disease (IBD) comprises two types of chronic intestinal inflammatory diseases, namely Crohn's disease (CD) and ulcerative colitis (UC) (196). The exact cause of this disease remains up to now unknown with increasing evidence that IBD results from an abnormal immune response to the GI microbiota in genetically susceptible hosts, together with defects in the intestinal epithelial barrier function (197,198). In UC inflammation typically affects the innermost layer of the mucosa of the large intestine. In UC Inflammation starts from the rectum and depending on severity of disease can affect the whole colon. With this features UC is differing from CD the other IBD entity. Here chronic inflammation mainly appears in the last part of the small intestine (terminal ileum) but can also affect parts of the large intestine and can actually affect the entire digestive system from mouth to anus and inflammation can extend to the entire thickness of the bowel wall. The symptoms of UC are quite diverse and range from abdominal pain, bloody diarrhea, rectal bleeding and urgency to defecate to fever, fatigue and weight loss (196,199). IBD is also well known to be associated with an increased risk for developing colorectal cancer (CRC) and other cancers secondary to long-lasting intestinal inflammation and immunosuppressive therapies (200–202). In UC it has been reported that the risk for CRC increases with duration of disease (203). But also a correlation between CRC risk and the location and extent of disease showed highest standardized incidence ratio (SIR) of 14.8 for pancolitis, followed by left-sided colitis (2.8) and proctitis (1.7) (204). Current therapies to treat UC are manifold but involve anti-inflammatory drugs and immunosuppressants like corticosteroids and TNF-alpha inhibitors (205,206). Proctocolectomy, the surgical removal of the large intestine including the anus, usually cures UC (207).

Shifts within the microbiota suggest that dysbiosis might also affect ulcerative colitis (208). Therefore FMTs have been investigated in various studies as an alternative approach for the treatment of patients with UC. Nevertheless recent studies show controversial results and varying response rates to FMT. Furthermore the factors influencing the efficacy of FMT in UC are still poorly understood (98).

To gain more insight into these unknown factors on the microbial side our aim was to investigate the microbiota of patients treated by repeated FMT and their corresponding donors to identify the microbial signature that correlates to clinical response.

Therefore we performed a prospective trial of repeated FMT in chronic active UC patients, in cooperation with Patrizia Kump and Christoph Högenauer form the Department of Gastroenterology and Hepatology at the Medical University of Graz, who designed and supervised the clinical study, providing fecal samples of patients and donors for subsequent microbiota analysis. Patients were treated with FMTs of healthy donors by colonoscopy after an antibiotic pre-treatment of 10 days. The transplantation was repeated every 14 days with FMTs from the same donor. We had 14 different donors for 17 patients only 2 donors donated for more than one patient. For subsequent microbiota analysis fecal samples were taken from donors and patients at each time point of FMT. Additionally we analyzed samples of UC patients at the beginning and the end of therapy. To analyze short term colonization we also took samples at day 3 after the first FMT. To control for the effects of the antibiotic pre-treatment a second group of 10 patients with chronic active UC receiving the antibiotic treatment without subsequent administration of FMT, were enrolled. Fecal samples for microbiota analysis were taken at four time points: At day -10, day 1, day 14 and day 90. For microbiota analysis we isolated the DNA from fecal samples, amplified the V4 region of the 16S rRNA gene and performed high throughput sequencing using Illumina MiSeq. The generated reads were pre-processed in mothur and further analyzed using QIIME (2).

Parts of the Results and Figures used are adopted according to the published manuscript: Kump, Wurm et al. Alimentary Pharmacology & Therapeutics, 2017 (2)

4.2. Materials and Methods

This methodological description has also been published in an original article (2).

4.2.1. Study Design

This is an open prospective trial of repeated FMT after antibiotic pre-treatment (FMT-group) with a nonrandomized control group with antibiotic pre-treatment only (AB-group) in chronic active UC patients. The study was conducted at the Medical University of Graz, Division of Gastroenterology and Hepatology and in a convent hospital in St. Veit an der Glan in Austria from July 2012 to July 2014. The trial (DRKS00005331 on DRKS) was approved by the ethics committee of the Medical University Graz (EK23-357ex10/11). Written informed consent was obtained from donors and patients, patients younger than 18 years required a parent's consent. All patients received antibiotic treatment including vancomycin 250mg qid (4 times a day), paromomycin 250mg tid (3 times a day) and nystatin 10ml (1 Million International Units) qid for 10 days (antibiotic pre-treatment). Subsequently 5 FMTs were performed by endoscopy as described below in 14 days intervals in the FMT-group (Figure 33). End of follow up was after 90 days. At each study visit the total Mayo score, fecal calprotectin and a standard laboratory analysis were assessed. The Mayo score is a disease activity index for UC summarizing the degree (from 0 to 3) of four important criteria: stool frequency, rectal bleeding, mucosal appearance at endoscopy and the physician's rating of disease activity. In summary the Mayo score can range from 0 points (no signs of activity) to a maximum of 12 points. The endoscopic Mayo score was initially assessed by two endoscopists and confirmed by an independent blinded endoscopist from electronic images of the site of most severe inflammation. Fecal samples for microbiota analyses were collected at each study visit (Figure 33). Primary end point was the reduction of the total Mayo score on day 90 (209). A reduction of the total Mayo score by $\geq$ 3 points was considered as a clinical response, whereas a drop of the Mayo score to $\leq$ 2 points was considered as remission. Patients with a response but no remission are denoted as partial responders. All clinical end point analyses were intention to treat (ITT). Patients who needed intensified therapy or terminated the study prematurely were assessed as treatment failures. Secondary

endpoints were to find a specific microbial signature in responders versus non responders and between donors by 16S rRNA gene sequencing. From June 2012 to July 2014 twenty-seven patients were recruited. An interim analysis performed in October 2013 including 15 patients who had passed their primary end point at day 90, revealed a significant reduction in the total Mayo score during the antibiotic pre-treatment without any FMT. From December 2013 to July 2014 10 further recruited patients were treated as controls receiving only antibiotics without consecutive FMT (AB-group), while 2 more patients of the FMT-group finished follow up (FMT-group, n=17). Follow up in the AB group was clinically and endoscopically assessed on day 1, 14 and 90 including additional microbiota analysis, fecal calprotectin and laboratory analysis. Due to a poor short- and medium-term tolerance of sole antibiotics in chronic active UC, further recruitment for this group was terminated (AB-group, n=10). Statistical analysis of clinical data was performed using SPSS version 15.0 (SPSS, Chicago, Illinois, USA). Categorical data were compared by Fisher's exact test, or the chi-square test, as appropriate; Student's t test was used for comparison of continuous variables, and the Mann-Whitney-U-test, if data were not normally distributed. P-values < 0.05 were considered statistically significant (2).

4.2.2. Study Population - Patients

Eligible patients with chronic active UC were aged between 16 and 80 years. Diagnosis was based on the current guidelines of the European Crohn`s and Colitis Organisation (210). Active ulcerative colitis was defined as a total Mayo score $\geq$ 4 and endoscopic subscore $\geq$ 1. All patients had treatment failures for at least one immunosuppressive therapy and/or anti-TNF-antibodies. Antibiotics and FMT were given as an add-on treatment to concomitant therapy provided that they were on a stable dose (no change in the dose of 5-ASA, immunosuppressant and anti-TNF therapy for 8 weeks, and a stable corticosteroid dose for at least two weeks) and still presenting with active disease. All concomitant therapies had to be continued at similar doses during the study. A tapering of steroids was allowed. Prior to antibiotic pretreatment coexisting infections with *Clostridium difficile* or any other enteric pathogens as *Salmonella, Shigella, Campylobacter, Yersinia,* and EHEC were excluded by culture and PCR (*C.difficile* toxin B gene). Patients were

excluded when they had any blood clotting disorder or treatments with oral anticoagulants, known allergies to the provided antibiotics or women who were pregnant (2).

4.2.3. Study Population - Donors

Donors were related or unrelated volunteers, had to be ≥ 18 years and were selected according to the Austrian guidelines for screening stool donors for FMT (211). Donors had to ensure that they had no antibiotic therapy or enteric infections within 3 month before stool donation. Eligible donors were allowed to donate feces for more than one patient but the same donor had to serve one patient throughout the 5 repeated FMTs. Donor screening was repeated every 6 month or sooner in the case of certain risks for infectious diseases (e.g. vacation in countries with a high risk for GI infections). 14 donors were eligible for the study. Two donors (donor no. 1 and 3) donated for more than one patient (donor no. 1 for A,B and C; donor no. 3 for E and K). The majority of the donors were unrelated, anonymous volunteers (n=6) or friends (n=3), 2 were partners and 3 were relatives. The mean age of the donor group was 38 years (range 27 – 54) and 62% were male (2).

4.2.4. Donor stool preparation and protocol for FMT

For each FMT fresh donor stool was collected in special vessels (2500 ml vessels for disposable specimen; LP Italiana Spa, Milano, Italy) and stored at 4º C. Donor stool preparation was performed under biohazard level 2 conditions according to the Austrian FMT guideline (211). Within 6 hours after donation a minimum of 50g of stool was diluted with sterile saline (200-500ml), homogenized and filtered in an one-step procedure using a standard household blender with integrated metal sieve. (Phillips HR 2084/90 Essence). A total of 250 to 500ml fecal suspension was placed into syringes for immediate application. An aliquot of the original donor stool was collected for microbiota analysis. Antibiotic pre-treatment was stopped 36 hours prior to the first FMT. The day following bowel preparation patients of the FMT group underwent total ileocolonoscopy. Overall 250 to 500ml of the fecal suspension was applied into the terminal ileum and the right colon after standard bowel preparation using a PEG based solution (Moviprep®, Norgine. Amsterdam,

Netherlands). In 14 days intervals 4 more repeated FMTs (day 14, day 28, day 42 and day 56) were performed by flexible sigmoidoscopy without any bowel preparation by deposing freshly prepared fecal suspension of the same donor into the left colon (2).

4.2.5. DNA extraction, 16S rRNA gene amplification and sequencing

Stool samples of patients and donors were immediately frozen and stored at -20°C. DNA extraction from stool samples was performed by mechanical lysis with a MagnaLyser Instrument (Roche Diagnostics, Mannheim, Germany) and subsequent total bacterial genomic DNA isolation with the MagNA Pure LC DNA Isolation Kit III (bacteria, fungi) in a MagNA Pure LC 2.0 Instrument (Roche Diagnostics) according to the manufacturer's instructions (112). For amplification of bacterial 16S rRNA gene the template-specific sequence 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), targeting the HVR V4 of the 16S rRNA gene were used (49). PCR reactions for each sample were performed in triplicates. Subsequently the amplicons were purified according to standard procedures, quantified, pooled and sequenced with the MiSeq Reagent Kits v3 (600 cycles, Illumina, Eindhoven, Netherlands) according to manufacturer's instructions with 20% OhiX (Illumina). The generated FASTQ files were used for microbiota analysis (2).

4.2.6. Microbiota analysis

Raw files from Illumina's MiSeq were processed according to the standard MiSeq SOP of mothur (53,55). Sequencing errors were reduced using mothur's pre.cluster command to remove sequences that arose due to sequencing errors. Chimeras were removed with UCHIME (57) and non-bacterial contaminants as chloroplasts and mitochondria were removed by classify.seqs and remove.lineage using the RDP training set (v.14). The high quality reads were aligned to the SILVA database (v.119) (56). For an OTU based analysis of the dataset, the processed fasta files from mothur were then introduced into QIIME version 1.8.0 (60). Open-reference OTU picking strategy was performed according to OTU similarity clustering with UCLUST (64) on a similarity score of 97% and Greengenes reference 13.08 was used (212). Subsequently diversity analyses

were performed in QIIME according to the core_diversity_analysis.py workflow. For the analyses samples were rarefied to at least 15376 sequences/sample. For statistical comparisons of alpha-diversity metrics (observed species, chao1, PD whole tree and Simpson's index) a nonparametric t-test with 999 Monte-Carlo permutations was performed and the Bonferroni method was used for multiple comparison corrections. Calculated beta diversity metrics (Bray Curtis, unweighted and weighted UniFrac) were compared by using the nonparametric ANOSIM measure. Significant differences in relative abundances of taxa were calculated by using a nonparametric Krukal-Wallis test using false discovery rate (FDR) correction and LEfSe (142). P-values below 0.05 were considered statistically significant (*$p<0.05$; **$p<0.01$; ***$p<0.001$). Presented values are always mean ± SEM if not indicated otherwise. Only samples of patients treated per protocol were included in the analysis (2).

4.3. Results

4.3.1. Clinical Outcomes

The study included a total of 27 patients with chronic active UC, all of them were included in the intention to treat analysis (ITT). The study was divided in two groups. One group included 17 patients that received an antibiotic pre-treatment with subsequent and repeated FMTs every two weeks. The second group was the control group, with 10 patients, which received the antibiotic treatment only (Figure 33) (2).

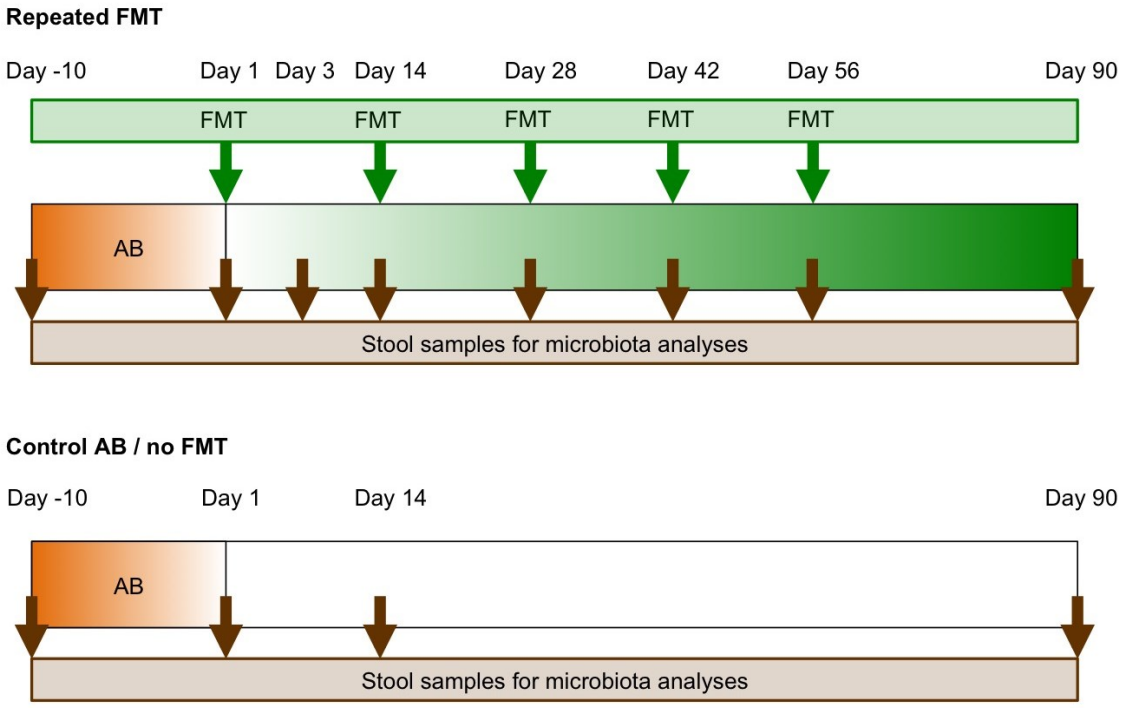


Figure 33. Study design. On the top is the scheme for the FMT-group, on the bottom for the AB-control group. Green arrows indicate when fecal microbiota transplantation was applied, brown arrows indicate when fecal samples for microbiota analysis were obtained. AB, antibiotics; FMT, fecal microbiota transplantation. Adopted from (2).

From the 17 patients, who received antibiotic pre-treatment and repeatedly performed FMT, 10 patients (59%) showed clinical response (Mayo score reduced by at least 3 points) to repeated FMTs on day 90. The remaining 41% showed no response to repeated FMTs at the final time point of clinical assessment. Within the group of responders, 6 patients, or 35% respectively, were classified as partial

responders and 4 patients (24%) achieved clinical remission (Total Mayo score of 2 points or less, Table 4). In contrast only 1 patient (10%) showed a partial response in the antibiotics control group at day 90 (Figure 34) (2).

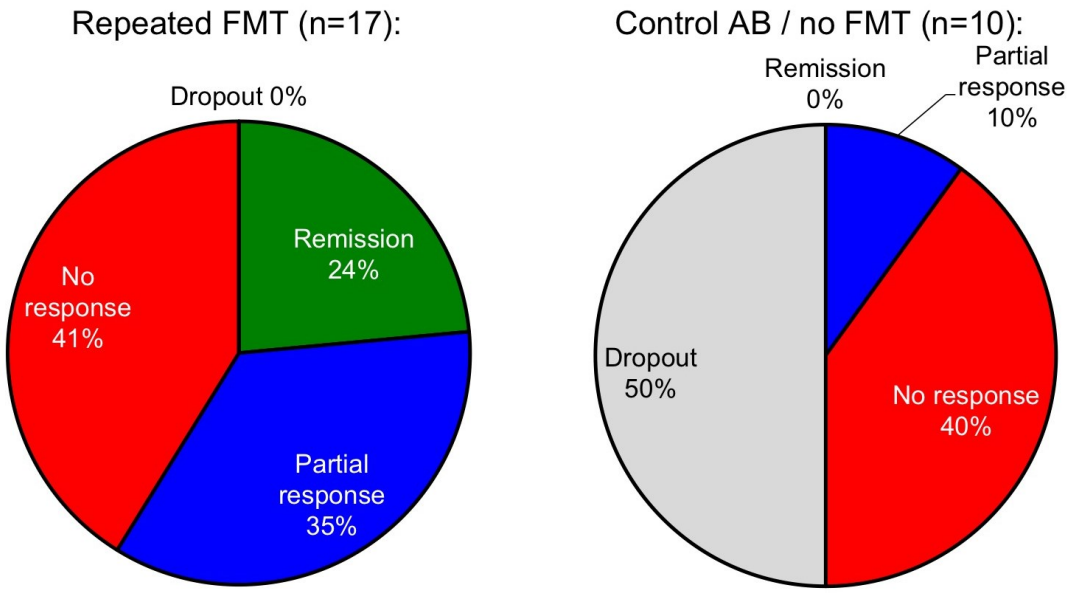


Figure 34. Clinical outcome based on the clinical response on day 90 at the end of therapy. Repeated FMT group (n=17) received 5 FMTs in 14 days intervals after 10 days of antibiotic treatment. The control antibiotics (AB) group received only antibiotics without further FMT treatment.

All 17 individuals from the FMT group, but only 8 from the control group finished the study. One dropped out of the study due to *Clostridium difficile* negative antibiotic associated diarrhea and one because of worsening of UC. From all 17 patients that finished the study in the FMT group, 13 were treated per protocol (PP) and included in the microbiota analysis. Within this group four patients reached clinical remission (RE), five showed a partial response (PR) and four patients showed no signs of response (NR). From the remaining four patients within the FMT group, three had deviating intervals between FMTs due to donor unavailability and one started self-medication with low dose steroids because of increasing stool frequencies after day 3. He was therefore considered as a non-responder. From all 8 patients that finished the study in the antibiotic control group, 5 were treated PP until the end of the study. One reached a partial response on day 90, the remaining four patients showed no response to the treatment. Another three patients in the antibiotic therapy group were tested positive for *C. difficile*, according to a positive culture test as well as a positive

Toxin-B qPCR assay after day 14. Therefore these patients were considered non responders and also not included to the microbiota analysis. Only patients who finished the study according to the protocol (PP) were considered for microbiota analysis. Patients within the FMT and within the AB control group show no significant differences beside gender. The baseline characteristics are given in Table 3. Number of patients that were treated per protocol and therefore included for microbiota analysis are shown in Figure 35 (2).

Table 3. Baseline characteristics of patients compared between FMT and AB group. Adopted from (2)

	FMT group (n=17)	**AB-group (n=10)**	**P value***
Mean age, years ± SD	44 ± 18	36 ± 13	0.20
Male sex, n (%)	14 (82)	3 (30)	**0.013**
Mean disease duration, years ± SD	8 ± 8	7 ± 6	0.53
Extent of disease, n (%)			
E1, proctitis	1 (6)	1 (10)	0.68
E2, left sided	10 (59)	7 (70)	
E3, pancolitis	6 (35)	2 (20)	
Concomitant drug treatment, n (%)	14 (82)	10 (100)	0.16
Mesalazine oral, n (%)	9 (53)	8 (80)	0.21
Immunosuppressants, n (%)	5 (29)	6 (60)	0.12
Anti-TNF, n (%)	1 (6)	2 (29)	0.44
Systemic corticosteroids, n (%)	10 (59)	3 (30)	0.063
Prior Immunosuppressants, n (%)	13 (76)	7 (70)	0.67
Prior anti-TNF, n (%)	10 (59)	4 (40)	0.29
Mean total Mayo score at inclusion ± SD	8.9 ± 1.6	8.1 ± 3.1	0.54
Endoscopic Mayo subscore at inclusion, n (%)			
Mayo 1	1 (6)	1 (10)	0.55
Mayo 2	5 (29)	3 (30)	
Mayo 3	11 (65)	6 (60)	
Disease severity by the total Mayo score at inclusion n (%)			
Mild (3-5 points)	1 (6)	2 (20)	0.33
Moderate (6-10 points)	13 (76)	6 (60)	
Severe (11-12 points)	3 (18)	2 (20)	

* Contingency tables were analyzed using Fisher's exact test or chi-square test (as appropriate), unpaired t-test for age, Mann-Whitney-U-Test for disease duration and for total Mayo score at inclusion.

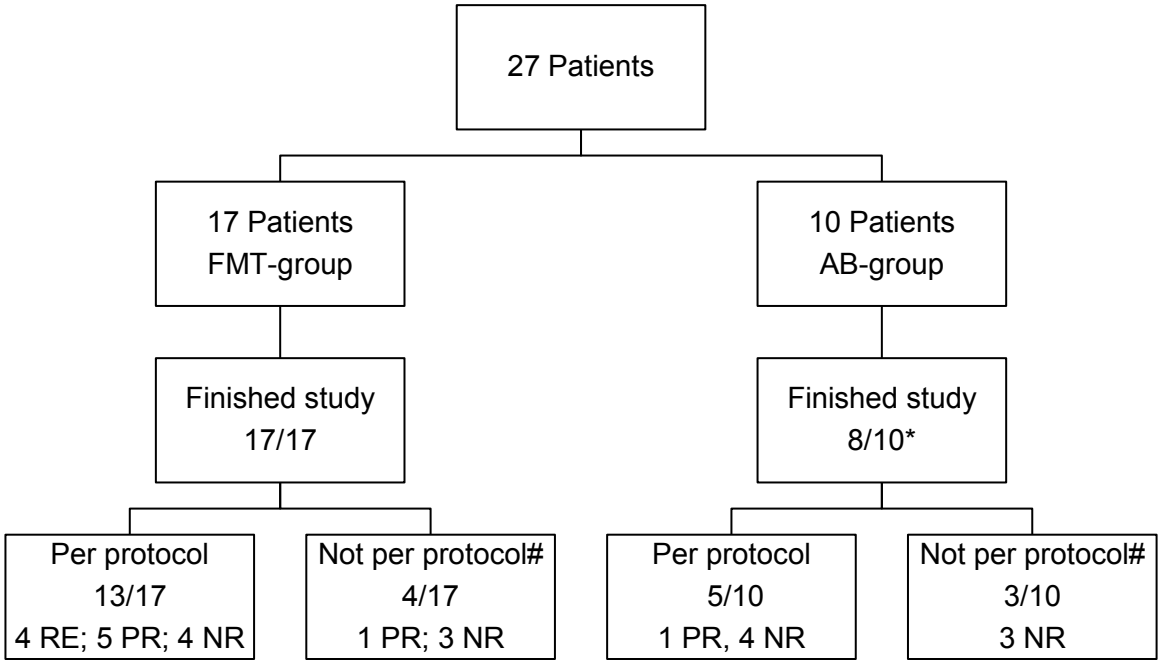


Figure 35. Number of patients in FMT and AB-control group included in study and treated per protocol. *2 patients in the AB-group did not finish the study, one because of worsening of ulcerative colitis and one due to antibiotic associated diarrhoea (*C. difficile* negative). # 3 patients in the FMT-group (1 PR, 2 NR) had prolonged faecal microbiota transplantation intervals due to donor unavailability between day 14 and day 56 respectively, 1 patient in the FMT- group started low dose steroids on his own because of increased stool frequency after day 3 and was considered as a non-responder. In the AB-group 3 patients were tested positive for *C. difficile* (positive culture and positive Toxin-B gene PCR-analysis) after day 14 and received further antibiotic treatment with Vancomycin 250mg qid for 10 days. Microbiota analysis was only performed in the patients treated per protocol who finished the study (AB, antibiotics; FMT, faecal microbiota transplantation; RE, remission; PR partial response; NR, no response). Adopted from (2).

An univariate analysis shows that treatment with FMT, a higher total Mayo score at baseline and male sex were predicting factors for response to treatment at day 90 (Table 5) (2).

Laboratory parameters of patients within the FMT group are given in Table 7. The values of C-reactive protein (CRP) values were significantly lower at day 90 in the responder and the remission group compared to those who achieved no clinical response (2).

In summary the FMT group tolerated the FMT treatment very well showing a tremendous clinical response. The antibiotic resulted in a slight temporary

improvement of disease, which didn't last without FMT and even resulted in a 50% drop-out rate.

Table 4. Patient characteristics according to FMT treatment response. Adopted from (2).

	No Response	**Response***	**Remission**	**p value§**	**p value ‡**
Number of patients n	7	10	4		
Total Mayo score at day -10, mean ± SD	8.1 ± 1.6	9.5 ± 1.4	9.3 ± 1.5	0.13†	0.37†
Endoscopic Mayo subscore at day -10, mean ± SD	2.3 ± 0.8	2.8 ± 0.4	2.5 ± 0.6	0.092	0,64
Total Mayo score at day 1, mean ± SD	6.6 ± 2.9	7.8 ± 2.0	6.3 ± 2.1	0.32	0.85
Endoscopic Mayo subscore at day 1, mean ± SD	2.0 ± 0.8	2.6 ± 0.7	2.0 ± 0.8	0.13	1.0
Total Mayo score at day 90, mean ± SD	8.0 ± 2.4	3.9 ± 3.1	0.5 ± 1.0	**0.013**	**0.0001**
Endoscopic Mayo subscore at day 90, mean ± SD	2.3 ±1.0	1.5 ± 1.3	0.3 ± 0.5	0.19	**0.004**
Δ total Mayo score with AB treatment, mean ± SD	1.6 ± 2.1	1.7 ± 2.0	3.0 ± 2.3	0.36	0.26
Age, years, mean ± SD	49 ± 22	41 ± 15	34 ± 11	0.38	0.25
Disease duration, years mean ± SD	9.7 ± 8.8	7.4 ± 8.1	5.8 ± 5.5	0.59†	0.44†
Concomitant drug treatment, n (%)					
Immunosuppressants	1/7 (14%)	4/10 (40%)	1/4 (25%)	0.78†	0.62†
Anti-TNF	0/7	1/10 (10%)	0/4	1.00†	1.00†
Systemic corticosteroids	4/7 (57%)	6/10 (60%)	2/4 (50%)	0.65†	0.65†
Extent of disease, n (%)					
Montreal E1	1/7 (14%)	0	0	0.34†	0.17†
Montreal E2	3/7 (43%)	7/10 (70%)	4/4 (100%)		
Montreal E3	3/7 (43%)	3/10 (30%)	0		
Disease severity by the total Mayo score at day -10, n (%)					
Mild (3-5 points)	1/7 (14%)	0	0	0.065†	0.54†
Moderate (6-10 points)	6/7 (86%)	7/10 (70%)	3/4 (75%)		
Severe (11-12 points)	0	3/10 (30%)	1/4 (25%)		

* including patients with partial response and remission; § between non responders and responders; ‡ between non responders and remission; Δ total Mayo score with AB treatment denotes the drop in the total Mayo score after the 10 day antibiotic pretreatment; † non-parametric test, day -10: before antibiotic treatment, day 1: before 1st FMT, day 90: end of study follow up.

Table 5. Univariate analysis for factors predicting treatment response and remission after treatment in the whole ITT population (n=27). Adopted from (2).

	Response		**p value**
	Yes (n=11)	**No (n=16)**	
Sex, m/f	10/1	7/9	**0.018**
Age, years, mean ± SD	42 ± 14	40 ± 18	0.78
Extent of disease, Montreal classification, E1/E2/E3	0/8/3	2/9/5	0.43
Duration of disease, years, median (range)	5.0 (1-28)	4.5 (1-22)	0.44
Mayo Score before treatment, median (range)	10 (8-12)	8 (4-12)	**0.037**
Endoscopic Mayo subscore before treatment, median (range)	3 (2-3)	2.5 (1-3)	0.082
Prior anti-TNF, y/n	7/4	7/9	0.44
Prior immunosuppressants, y/n	7/4	13/3	0.39
Ongoing anti-TNF, y/n	2/9	1/15	0.55
Ongoing immunosuppressants, y/n	5/6	6/10	0.71
Ongoing corticosteroids, y/n	5/6	8/8	1.0
Ongoing 5-ASA, y/n	9/2	8/8	0.12
FMT, y/n	10/1	7/9	**0.018**
	Remission		
	Yes (n=4)	**No (n=23)**	
Sex, m/f	3/1	14/9	0.52
Age, years, mean ± SD	34 ± 11	42 ± 17	0.37
Extent of disease, Montreal classification, E1/E2/E3	0/4/0	2/13/8	0.25
Duration of disease, years, median, (range)	4.5 (1-13)	5 (1-28)	0.58
Mayo Score before treatment, median, (range)	9 (8-11)	9 (4-12)	0.63
Endoscopic Mayo subscore before treatment, median (range)	2.5 (2-3)	3 (1-3)	0.69
Prior anti-TNF, y/n	2/2	12/11	0.67
Prior immunosuppressants, y/n	3/1	17/6	1.0
Ongoing anti-TNF, y/n	0/4	3/20	1.0
Ongoing immunosuppressants, y/n	1/3	10/13	0.62
Ongoing corticosteroids, y/n	1/3	12/11	0.60
Ongoing 5-ASA, y/n	2/2	15/8	0.61
FMT, y/n	4/0	13/10	0.26

Table 6. Laboratory parameters in patients according to FMT treatment response. Adopted from (2).

	No Response	Response*	Remission	p value§	p value‡
Number of patients (n)	**7**	**10**	**4**		
Laboratory values at day -10					
WBC (×10^9/L)	11.9 ± 3.0	8.4 ± 3.2	7.1 ± 1.9	0.057	**0.023**
Hemoglobin g/dL	13.6 ± 1.8	13.8 ± 1.1	13.9 ± 1.8	0.86	0.81
Thrombocytes (×10^9/L)	361 ± 82	297 ± 94	277 ± 126	0.19	0.23
CRP mg/L	7.0 ± 4.3	9.5 ± 12.0	3.6 ± 4.2	0.96†	0.29†
Fecal calprotectin, µg/g	380 ± 127	1317 ± 469	1244 ± 342	**0.013**	0.15
Laboratory values at day 1					
WBC (×10^9/L)	9.8 ± 2.7	7.0 ± 2.5	5.1 ±1.0	**0.044**	**0.003**
Hemoglobin g/dL	13.6 ± 2.3	13.2 ± 1.3	13.0 ± 2.1	0.68	0.69
Thrombocytes (×10^9/L)	341 ± 86	252 ± 59	260 ± 91	**0.023**	0.17
CRP mg/L	13.5 ± 15.0	4.1 ± 5.2	2.0 ± 2.1	0.27†	0.18†
Fecal calprotectin, µg/g	406 ± 258	664 ± 603	254 ± 270	0.34	0.40
Laboratory values at day 90					
WBC (×10^9/L)	8.6 ± 3.7	6.6 ± 1.9	5.4 ± 1.6	0.26	0.15
Hemoglobin g/dL	13.5 ± 1.8	13.3 ± 1.3	13.3 ± 2.0	0.73	0.83
Thrombocytes (×10^9/L)	352 ± 110	276 ± 58	280 ± 73	0.09	0.29
CRP mg/L	21.7 ± 20.4	4.1 ± 6.3	1.8 ± 1.3	**0.022†**	**0.033†**
Fecal calprotectin, µg/g	867 ± 593	552 ± 622	309 ± 394	0.34	0.14

all values are means ± SD; * including patients with partial response and remission; § between non responders and responders; ‡ between non responders and remission; p-values were obtained by unpaired t-test, p-values indicated by † were obtained by Mann Whitney-U-test; day -10: before antibiotic treatment, day 1: before 1st FMT, day 90: end of study follow up.

4.3.2. Microbiota analysis - Differences in the microbiota of patients and donors according to treatment response

Figure 36 is indicating the taxonomic profile on family level of the patients' fecal microbiota pre-AB, pre-FMT and at the end of therapy (day 90) for non-responders, partial responders and remission in the FMT group and day 90 in the AB only control group. The taxonomic profiles of the donor fecal samples are stratified according to treatment response in UC patients. Amongst others the relative abundance of *Enterobacteriaceae* (red) increased directly after antibiotic pre-treatment. Additionally *Verrucomicrobiaceae* (yellow) is increased in donor samples leading to clinical response in UC patients (2).

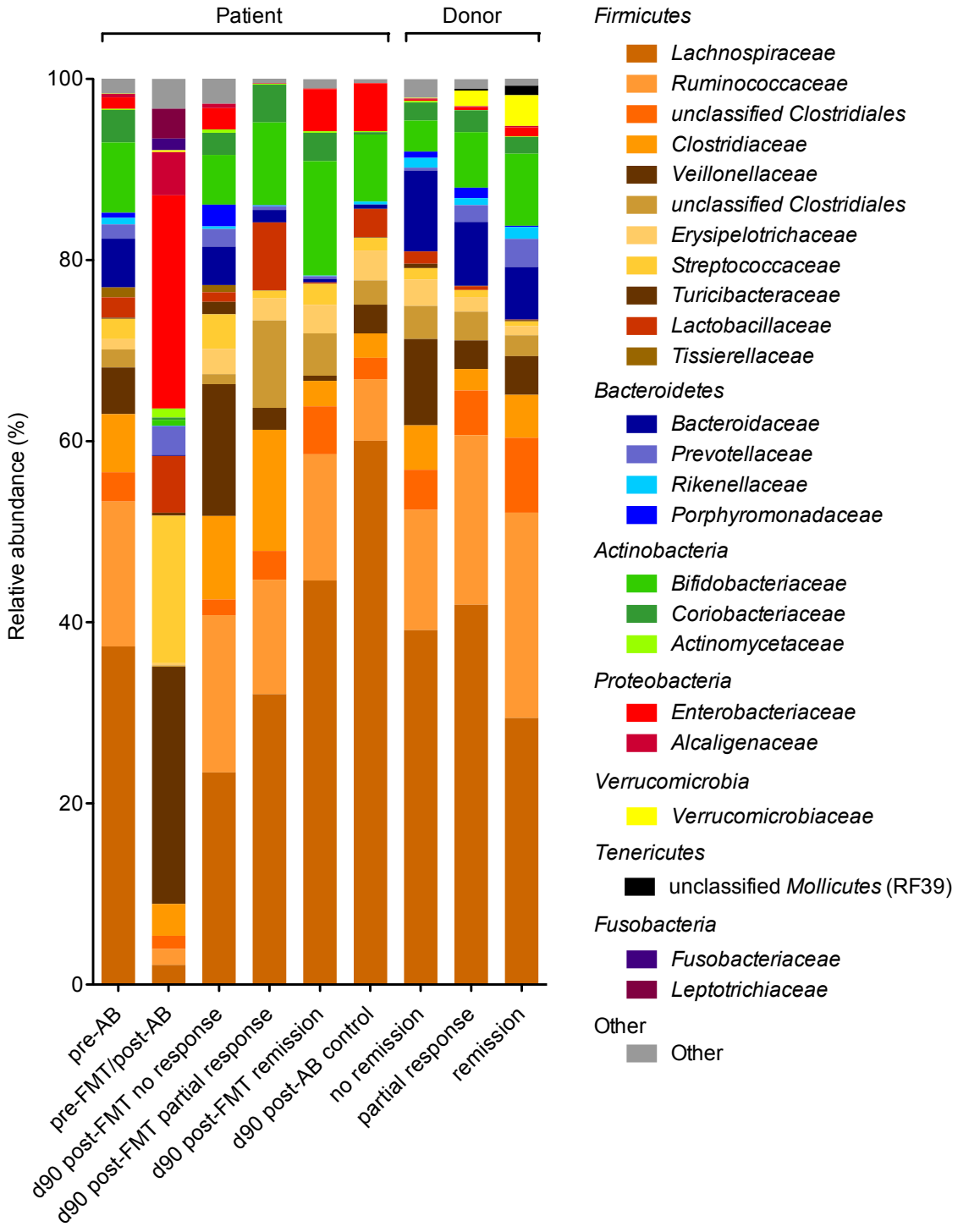


Figure 36. Taxonomic composition of the patient and donor microbiota at different time points during the study. Patient and donor microbiota composition are based on family level and are presented according to treatment response. Adopted from (2).

Based on the clinical outcome we tried to identify, if efficacy and failure of repeated FMTs in chronic active UC may be associated to specific microbial

compositions. In patients' samples there was no significant difference in richness evident. Neither in patients that reached clinical remission nor in patients without clinical response (Figure 37 A). PCoA based on the phylogenetic measure unweighted UniFrac indicated significantly different recipient microbiotas in NR vs RE ($p<0.001$) although separation of both groups was rather weak (ANOSIM R=0.2386) (Figure 37 B) (2).

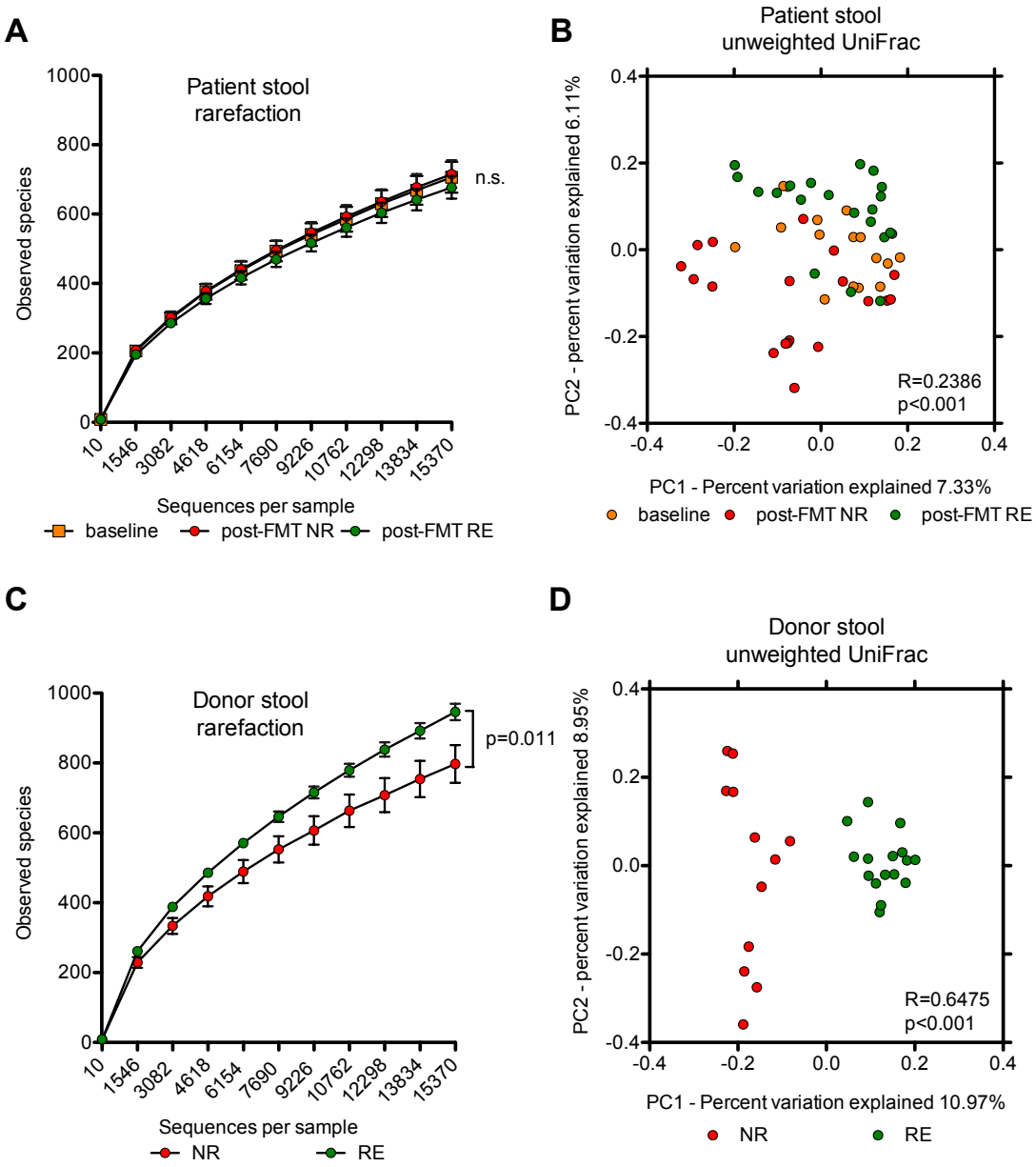


Figure 37. Microbial composition of the donor stools determines efficacy of FMT in chronic active UC. A: No significant difference in microbial richness (observed species) of stool samples from recipients pre- and post-FMT when stratified according to treatment response (orange: baseline at day -10, n=16; red: post- FMT no response (NR), n=19; green: post-FMT remission (RE), n=21). B: Principle coordinates analysis (PCoA) of unweighted UniFrac distance shows a

significant clustering of patient stools comparing baseline samples (at day -10; orange) and post-FMT (red: NR; green: RE). The low R-value=0.2386 indicates an overall weak grouping. C: Donor stools leading to remission in recipients showed a significantly increased richness compared to donor stools leading to no response (red: NR, n=12; green: RE, n=16; p=0.011). Plots show mean ± SEM, p-values given for difference at 15376 sequences per sample based on two-sample t-test. D: PCoA indicated significantly different microbial community types with a strong grouping when donor stools were stratified according to response in the recipient (red: NR, n=12; green: RE, n=16; p<0.001, R=0.6475, ANOSIM). Adopted from (2).

Next we assessed the transplant. Importantly, microbiota of donors that lead to remission (RE), meaning a total Mayo score of 2 or less at day 90, showed clear differences in microbial richness (observed species, p=0.011) and significant separation in a PCoA Plot based on unweighted UniFrac (R= 0.6475, p<0.001; ANOSIM) compared to donor stools that were unable to cause a response (NR), showing a decrease of the total Mayos by only 2 points or less on day 90 compared to baseline (Figure 37 C&D, Figure 38) (2).

The microbiota of donor stools which lead to a partial response (PR), showed a non-significant trend towards increased richness and alpha diversity compared to donor stools leading to NR. The alpha diversity metrics observed species, Chao 1, PD whole tree and Shannon index were significantly increased in donor stools leading to RE compared to NR (Figure 38) (2).

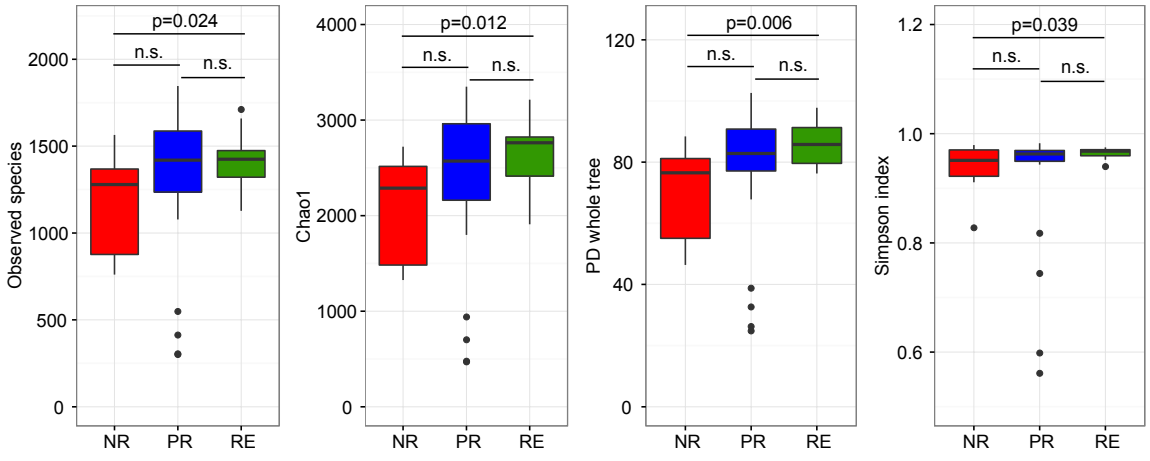


Figure 38. Microbial diversity of donor stools stratified according to patient's FMT response. A statistically significant difference was observed between donors inducing RE compared to donors inducing PR or NR. The observed species, Chao1, PD whole tree and Simpson indices are shown (red: NR; blue: PR; green:

RE; n=12-23; 32672 reads/sample; nonparametric t-test, 999 Monte Carlo permutations, Bonferroni post-test). Adopted from (2).

Although 5 patients showed clear clinical response and improvement classified as PR according to the study's guidelines, there is no significant difference based on microbial alpha diversity neither compared with donor stool leading to RE nor with donor stool that shows no response in patients. This explains that pooled donor stool according to clinical response (PR and RE) leads to loss of significance in microbial richness compared to donor stool without clinical response (NR) (Figure 39) Additionally it also blurs the separation of both groups with a PCoA (

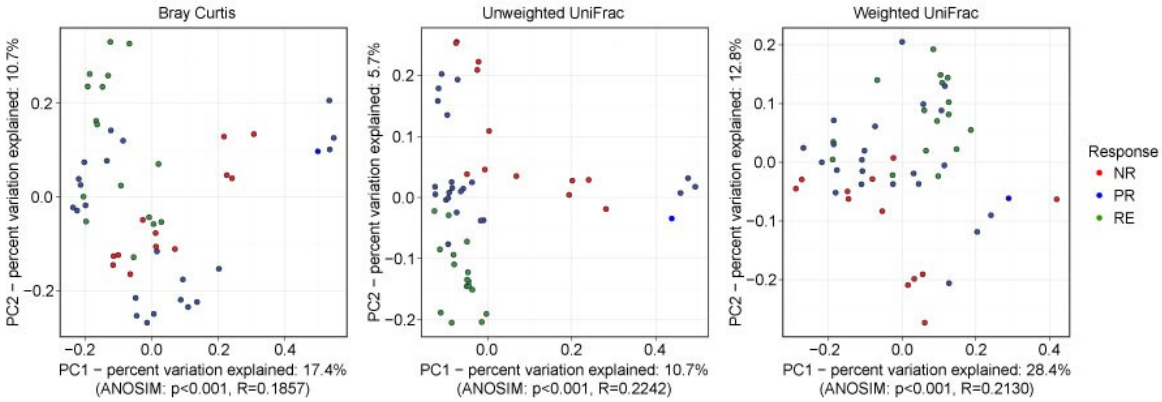


Figure 40, Figure 41) (2). For subsequent taxonomic profile analysis we therefore compared donor stool samples that caused remission (RE) with those leading to no response (NR) in patients to find the most promising strains that are capable to counteract inflammation in patients with UC.

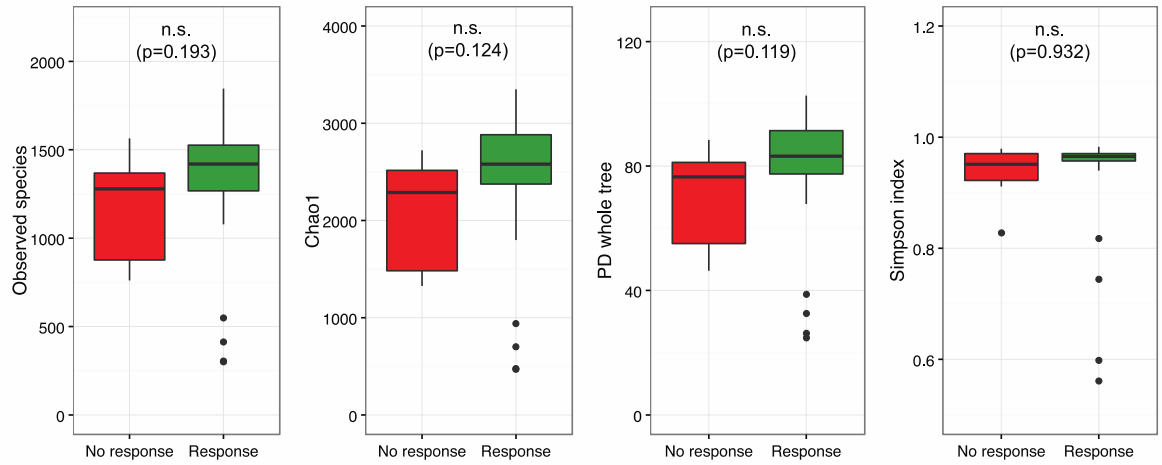


Figure 39. Alpha diversity and species richness: Non-responders vs Responders. No significant differences in all tested alpha diversity measurements for fecal microbiota between donor samples leading to clinical response vs no response. (red: no response, n=12; green: response, n=39; p-values given for

difference at 15376 sequences per sample ; nonparametric t-test, 999 Monte Carlo permutations, Bonferroni post-test).

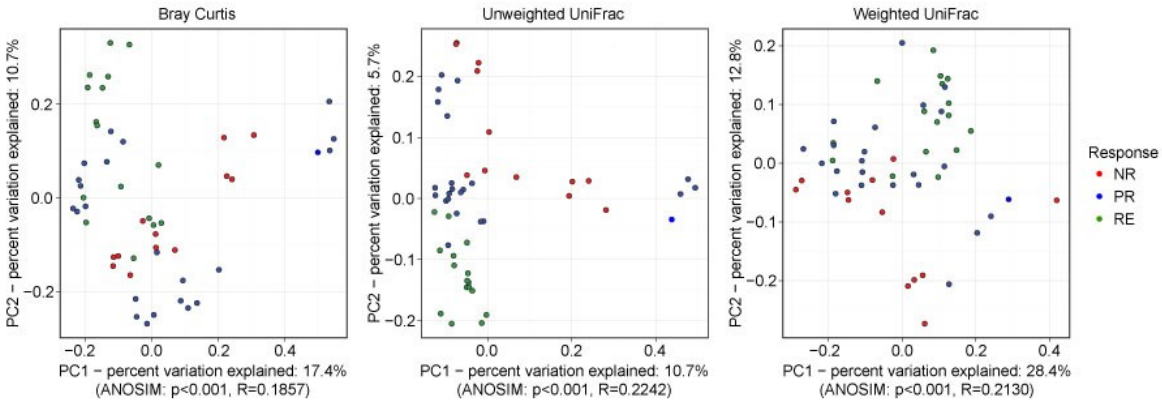


Figure 40. Donor microbiotas grouped according to FMT treatment response: Remission vs partial response vs no response. Dissimilarity of the donor´s microbiota analyzed by Bray Curtis distance (left panel), unweighted UniFrac (medium panel) and weighted UniFrac (right panel) distance. Red dots: no response, NR; blue dots: partial response, PR; green dots: remission, RE. (n=12-23; ANOSIM). Adopted from (2).

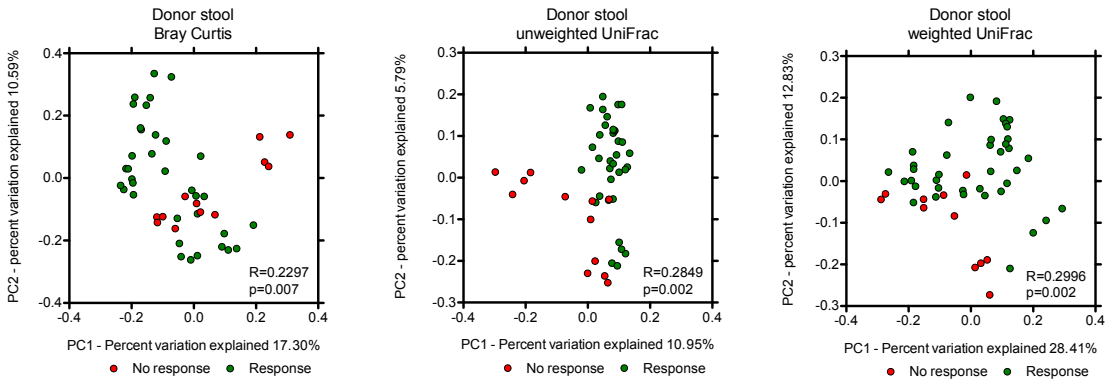


Figure 41. Donor microbiotas grouped according to FMT treatment response: Response vs no response. Dissimilarity of the donor´s microbiota analyzed by Bray Curtis distance (left panel), unweighted UniFrac (medium panel) and weighted UniFrac (right panel) distance. Red dots: no response, NR; green dots: response, RE. (n=12-39; ANOSIM).

In Figure 42, Figure 43 and Figure 44 all significant taxonomic differences that are present between donor microbiota that lead to remission and donor microbiota that show no response in patients are illustrated. The results are stratified according to their mean relative abundance into high (>3%), low (3%-0.1%) and ultralow abundant taxa (<0.1%). This should simplify the representation of significantly different abundant taxa between RE and PR but has also biological relevance. High abundant taxa e.g. are more likely to produce more bacterial metabolites and

tend to represent a larger bacterial surface including potential antigenic molecules in total compared to lower abundant species. Nonetheless low abundant species can provide essential genetic information to the microbiome, resulting healthy or disease conditions. Therefore high as well as low or ultralow abundant taxa can represent a keystone species for the gut microbiota (2).

The most prominent taxa we found in the high abundant category and were significantly different in donor stool that caused RE compared to donor stool leading to NR were: *Actinobacteria*, *Ruminococcaceae*, *Ruminococcus* spp. and *Akkermanisa muciniphila*. They were significantly increased in FMT that lead to RE in UC patients, with a mean relative abundance of more than 3% (Figure 42). Especially the finding of *A. muciniphila* shows almost a presence/absence finding in the two groups indicated by the mean relative abundance of 3.32±0.8% in the RE donor stool group compared to 0.08±0.07% in the NR donor stool group. *A. muciniphila* was not present in 5/12 samples from the NR donor stool group. These significant differences were obtained using two separate test. We compared relative abundance values using a nonparametric Kruskal-Wallis test with false discovery rate (FDR) correction implemented in QIIME. Additionally we used the linear discriminant analysis effect size (LEfSe) method by Segata et al. encoding biological consistency and effect relevance (142). In the range of low abundant bacteria (3%-0.1%) the donor microbiota leading to RE showed significantly enriched *Mollicutes*, whereas *Paraprevotellaceae*, *Erysipelotrichaceae*, *Veillonella dispar* and *Eubacterium* were increased in NR donor stool (Figure 43). In the ultralow abundant taxa group (below 0.1% relative abundance), an unclassified genus within the family of the *Rikenellaceae* was increased in NR donor stool, whereas several taxa within the class *Clostridia* were significantly enriched in RE donor stool. These included unclassified *Christensenellaceae*, *Mogibacteriaceae* and *Ruminococcaceae* as well as *Dehalobacterium* spp (2).

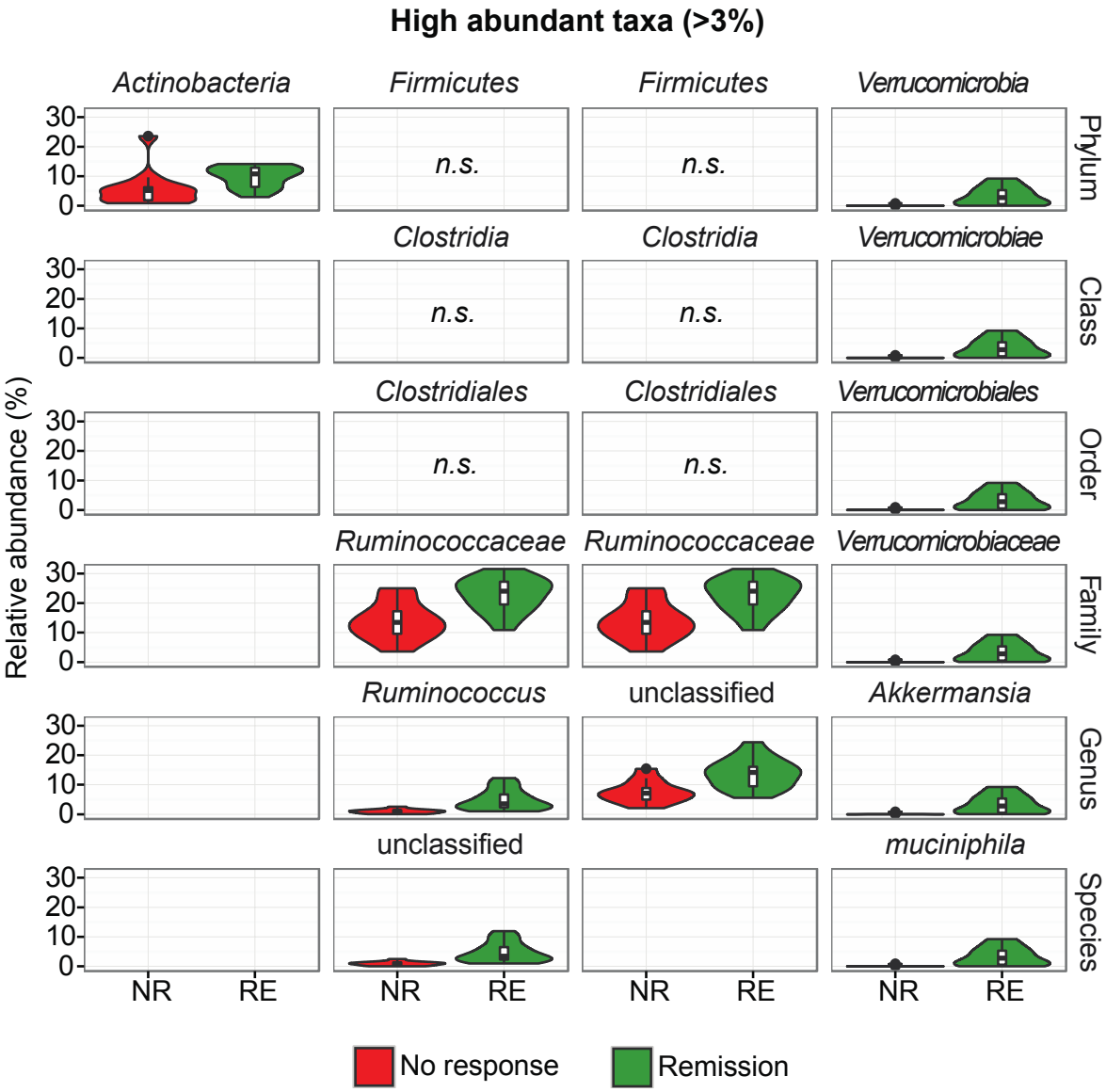


Figure 42. Taxonomic signature in donor stools associated with treatment response. Significantly different taxa from phylum to species level in the donors' microbiota associated to NR and RE in the recipient according to high abundant taxa (mean >3%). Plotted taxa show are significantly different between both treatment response groups based on a non- parametric Kruskal-Wallis test with FDR correction in QIIME ($p<0.05$) as well as on discriminatory features calculated by LEfSe ($p<0.05$ and LDA-score > 2; red: no response, NR, n=12; green: remission, RE, n=16). Adopted from (2).

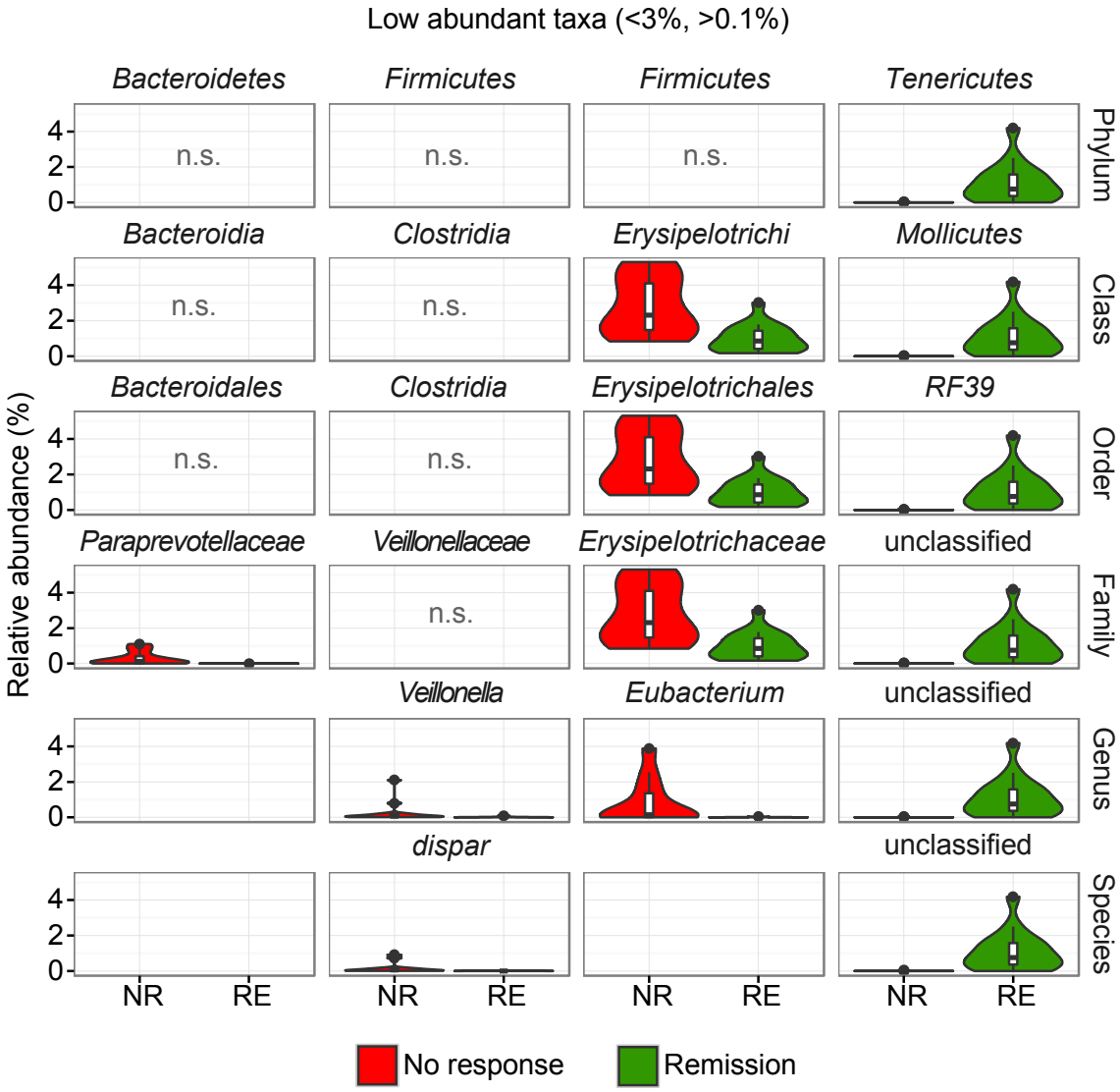


Figure 43. Low abundant taxa in donor stools associated with treatment response. Significantly different taxa from phylum to species level in the donors' microbiota associated to NR and RE in the recipient according to low abundant taxa (mean <3% and >0.1%). Plotted taxa show are significantly different between both treatment response groups based on a non- parametric Kruskal-Wallis test with FDR correction in QIIME ($p<0.05$) as well as on discriminatory features calculated by LEfSe ($p<0.05$ and LDA-score > 2; n=12-16; red: no response, NR; green: remission, RE). Adopted from (2).

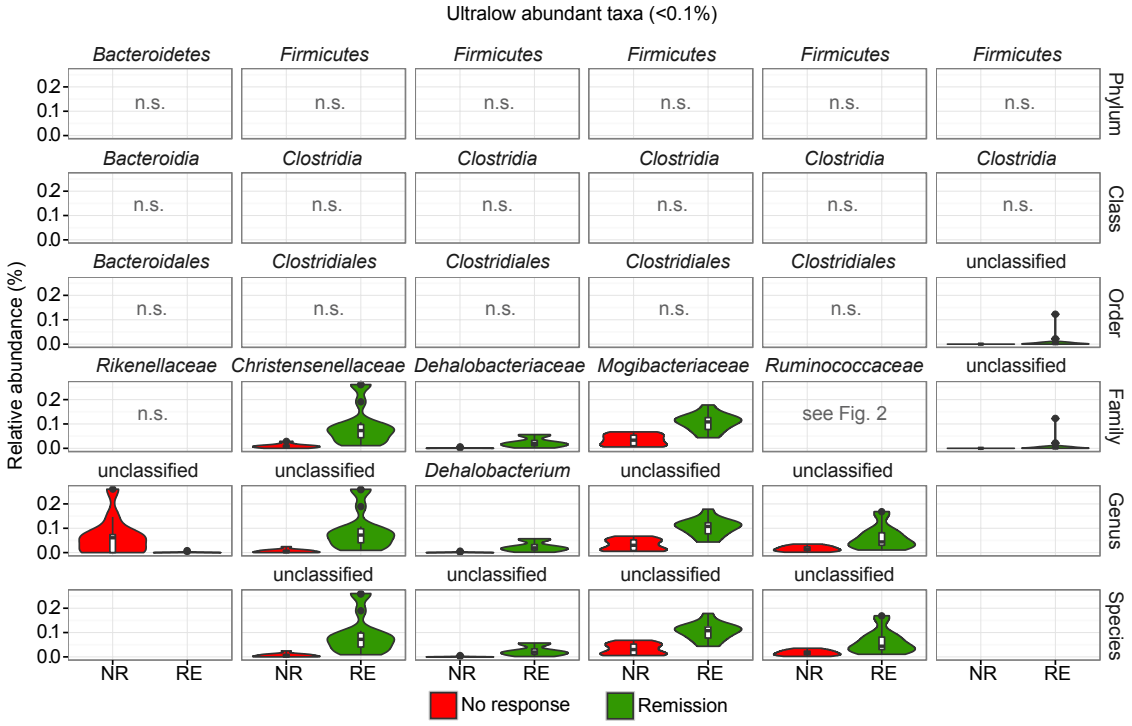


Figure 44. Ultralow abundant taxa in donor stools associated with treatment response. Significantly different taxa from phylum to species level in the donors' microbiota associated to NR and RE in the recipient according to ultralow abundant taxa (<0.1%). Plotted taxa show are significantly different between both treatment response groups based on a non- parametric Kruskal-Wallis test with FDR correction in QIIME ($p<0.05$) as well as on discriminatory features calculated by LEfSe ($p<0.05$ and LDA-score > 2; n=12-16; red: no response, NR; green: remission, RE). Adopted from (2).

High levels of *Ruminococcus gnavus* and *R. torques* could already be linked to the mucosa associated microbiota in UC compared to healthy controls (213). *R. gnavus* was also found at high levels in donors that do not lead to a response in chronic UC patients after FMT (214). Here *Rumminococcaceae*/*Ruminococcus* showed a strong increase in donor stools leading to RE. Therefore we subjected the 16 most prominent sequences that were assigned to the family *Ruminococcaceae*, to BLAST analysis against the NCBI database for "16S rRNA sequences (Bacteria and Archaea)" (Figure 45). This analysis shows that no sequence shares a high coverage with *R. gnavus* or *R. torques*. Most OTUs that were assigned as *Ruminococcus* spp. show the highest identity with *R. bromii*, *R. champanellensis* and *R. callidus*. Other sequences assigned as Ruminococcaceae could be assigned as *Faecalibacterium prausnitzii*. In summary the data shows

that neither *R. gnavus* nor *R. torques* were enriched in donor stool that lead to remission in patients. In contrast other species from genus *Ruminococcus*, such as *R. bromii* and *R. champanellensis,* might have beneficial effects for UC patients when they are administered via FMT (2).

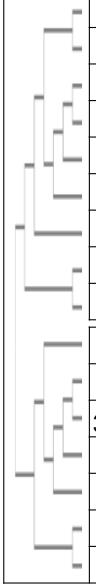
MAFFT	OTU	Genus (Qiime)	NCBI BLAST, best identity	RE (r.a.)	NR (r. a.)
	146554	*Ruminococcus*	*R. Bromii*, 99%	2.51%	0.64%
	163243	*Ruminococcus*	*R. Bromii*, 98%	0.34%	0.00%
	181961	*Ruminococcus*	*R. champanellensis*, 97%	0.41%	0.00%
	323135	*Ruminococcus*	*R. callidus*, 95%	0.36%	0.00%
	48084	*Ruminococcus*	*R. champanellensis*, 97%	0.24%	0.00%
	147969	*Ruminococcus*	*R. champanellensis*, 91%	0.40%	0.00%
	183439	unclassified	[*Clostridium*] *celleobioparum*, 93%; (*R. Albus,* 89%)	1.08%	0.02%
	363017	unclassified	[*Clostridium*] *celleobioparum*, 91%; (*R. Bromii,* 88%)	0.28%	0.01%
	358781	unclassified	*R. albus*, 90%	1.77%	0.02%
	185575	unclassified	*F. Prausnitzii*, 95%	2.04%	1.52%
	190171	unclassified	*Gemmiger formicilis*, 99%; (*F. Prausnitzii*, 93%)	1.33%	0.51%
	3236435	unclassified	*Gemmiger formicilis*, 98%; (*F. Prausnitzii*, 93%)	0.26%	0.18%
	265871	unclassified	*F. Prausnitzii*, 98%	1.15%	1.88%
	198956	*Faecalibacterium*	*F. Prausnitzii*, 98%	2.03%	1.21%
	185763	*Faecalibacterium*	*F. Prausnitzii*, 99%	0.45%	1.20%
	185390	*Faecalibacterium*	*F. Prausnitzii*, 99%	0.36%	0.23%

Figure 45. Most prevalent OTU sequences within family *Ruminococcaceae* of donor stool samples leading to remission. The 16 most prevalence OTUs in donor samples leading to RE were blasted against the 16S rRNA sequences (Bacteria and Archaea) database (update date: 2017/03/16; number of sequences: 18590) based on best identity. Phylogram was created using online multiple sequence alignment, MAFFT (215). Relative abundances (r. a.) of *R. bromii* and *R. champanellensis* OTUs show differences in donor stools leading to remission compared to donor stools with no response. This is not evident for *F. prausnitzii* OTUs. Adopted from (2).

As stated earlier *A. muciniphila* is almost absent in NR donor stool and one of the most prominent differences between NR and RE donor samples. Interestingly *A. muciniphila* is also almost absent in all UC patient samples at baseline which might contributes to the definition of dysbiosis in chronic active UC (Figure 46 A). *A. muciniphila* was dramatically increased 3 days after the first FMT reaching a significant difference at day 3 in patients that achieved RE compared to patients showing a PR or NR at day 90. In follow-up samples *A. muciniphila* was not detectable in any of the patients regardless of clinical outcome, indicating that *A. muciniphila* is able to achieve short- but no long-term colonization exceeding 14

day intervals in patients (Figure 46 B). Additionally *Dialister* sp. was associated with patients going into remission. Antibiotic therapy in UC patients (post-AB / pre-FMT) lead to a significant decrease of the relative abundance of *Dialister* sp. compared to baseline samples of UC patients. While the rel. abundance of *Dialister* sp. increased again in UC patients' post-FMT in the NR and PR group an showed no difference to baseline, the rel. abundance of *Dialister* sp. was still significantly lower in samples of RE patients post-FMT (Figure 46C) (2).

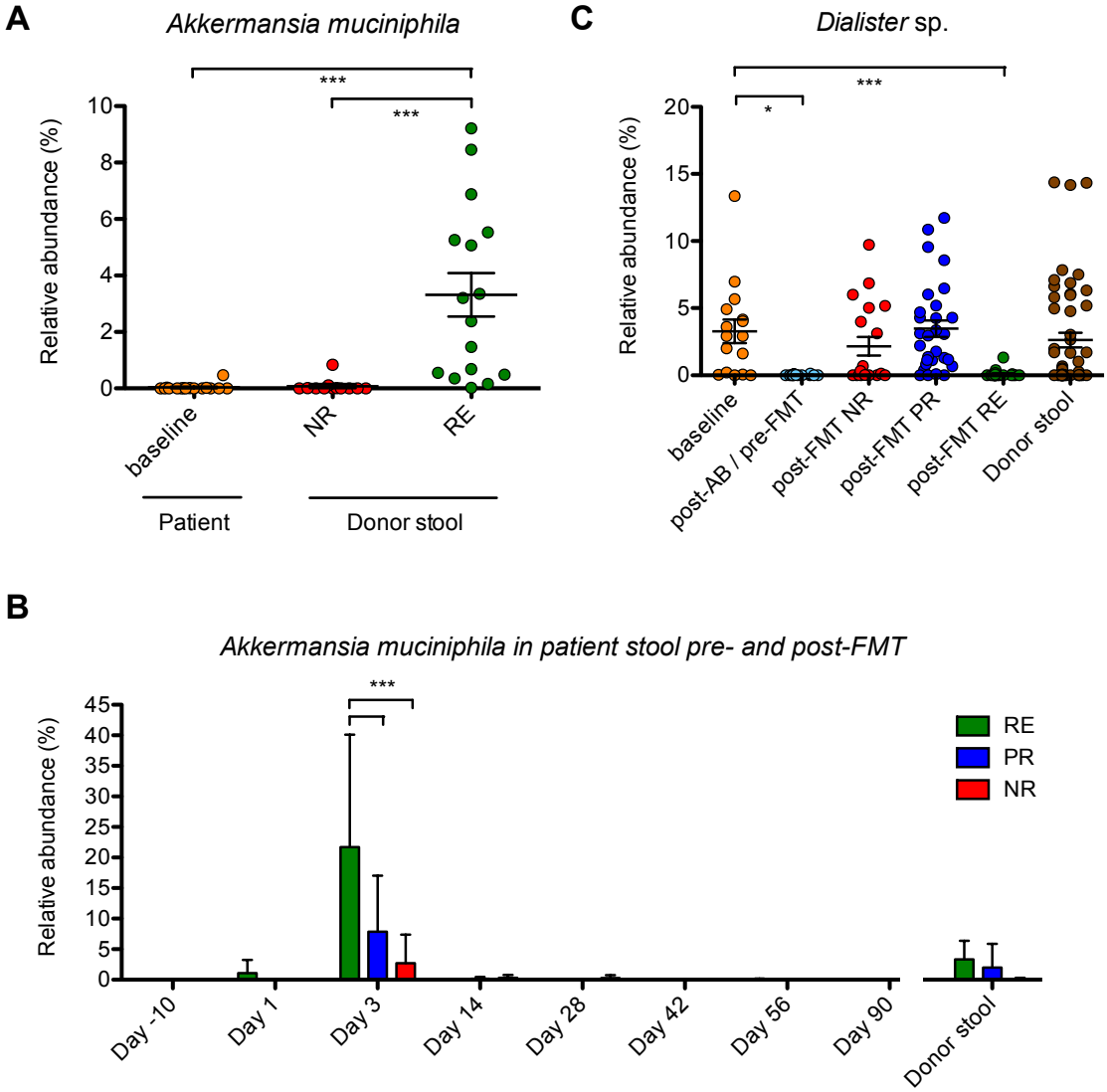


Figure 46. Efficacy of FMT correlates to abundance of specific taxa in donors and patients after FMT. A: Relative abundance of *A. muciniphila* in patient stools pre-FMT (orange) and donor stools according to treatment success of FMT (red:

no response, NR; green: remission, RE; Kruskal-Wallis test, Dunn's multiple comparison test, ***p<0.001). B: A high relative abundance of *A. muciniphila* was detected in patients responding to FMT on the first day after the initial FMT application (day 3), with a significant increase in RE patients compared to partial response (PR) or NR. In contrast *A. muciniphila* abundance was low or non-detectable before FMT (day-10, day 1). No long-term colonization of *A. muciniphila* was seen in the other post-FMT samples (day 14, 28, 42, 56 and 90) from UC patients (red: NR; blue: PR; green: RE; n=2-7, Two-way ANOVA, Bonferroni posttest, ***p<0.001). C: Relative abundance of *Dialister* sp. in stools of UC patient at baseline (orange, n=16), post-antibiotic treatment (light- blue, n=22) and post-FMT according to treatment response (d3 to d90; red: NR, n=19; blue: PR, n=29; green: RE, n=21) and in donor stools (brown, n=51, Kruskal-Wallis test, Dunn's multiple comparison test, **p<0.001, ***p<0.001). Adopted from (2).

According to the patients' fecal microbiota, clinical response to treatment is not associated with long-term colonization of *A. muciniphila*. Alternatively *A. muciniphila* could be a marker for an overall healthy fecal microbiota for effective FMT in chronic active UC. To determine differences in the microbiota composition of *A. muciniphila* positive and negative fecal samples, we analyzed 17 donor stool samples without *A. muciniphila* and compared it to 17 donor stool samples with the highest relative abundance of *A. muciniphila* (>1.5% rel. abundance), independent from the clinical outcome in patients. We excluded all OTUs that were assigned to *A. muciniphila* from the dataset prior to analysis. According to LEfSe analysis (LDA score >4.0) an *A. muciniphila* dominant microbiota was in addition to higher abundance of *Ruminococcus* and *Coprococcus* also enriched with *Bifidobacterium adolescentis*. A negative association with *Blautia* spp. could also be detected (Figure 47).

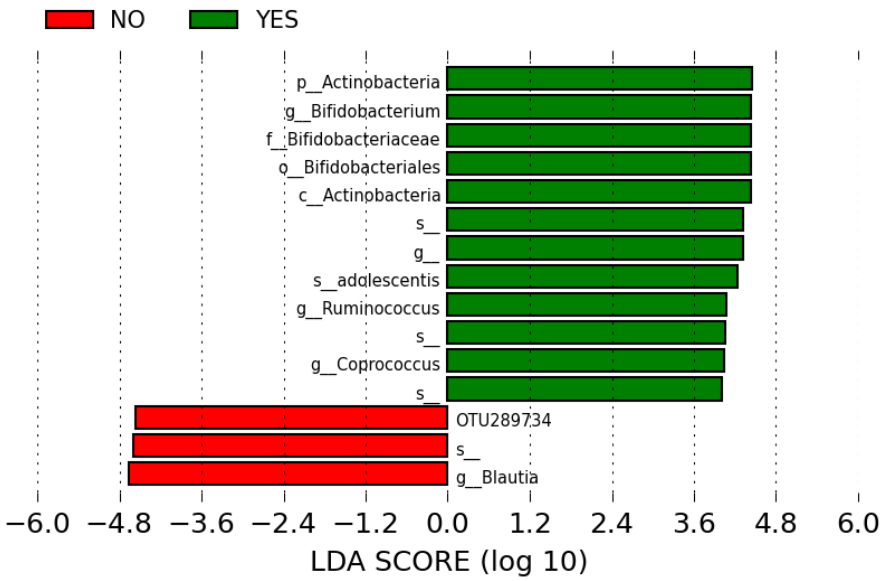


Figure 47. Taxa associated to a *A. muciniphila* dominated microbiota. Donor stool samples stratified into groups *A. muciniphila* positive samples (rel. abundance >1.5%, green "YES", n=17) and *A. muciniphila* negative samples (rel. abundance = 0%, red "NO", n=17). After extracting *A. muciniphila* assigned OTUs from the dataset groups were tested with LEfSe. Results indicate significant different taxa based on a LDA score of 4.0 or higher.

According to a nonparametric Spearman correlation test the rel. abundance of *Bifidobacterium* spp. and *Bifidobacterium adolescentis,* respectively, significantly correlated to the rel. abundance of *Akkermansia muciniphila* in all 51 donor stool samples tested, independent of clinical response (Figure 48).

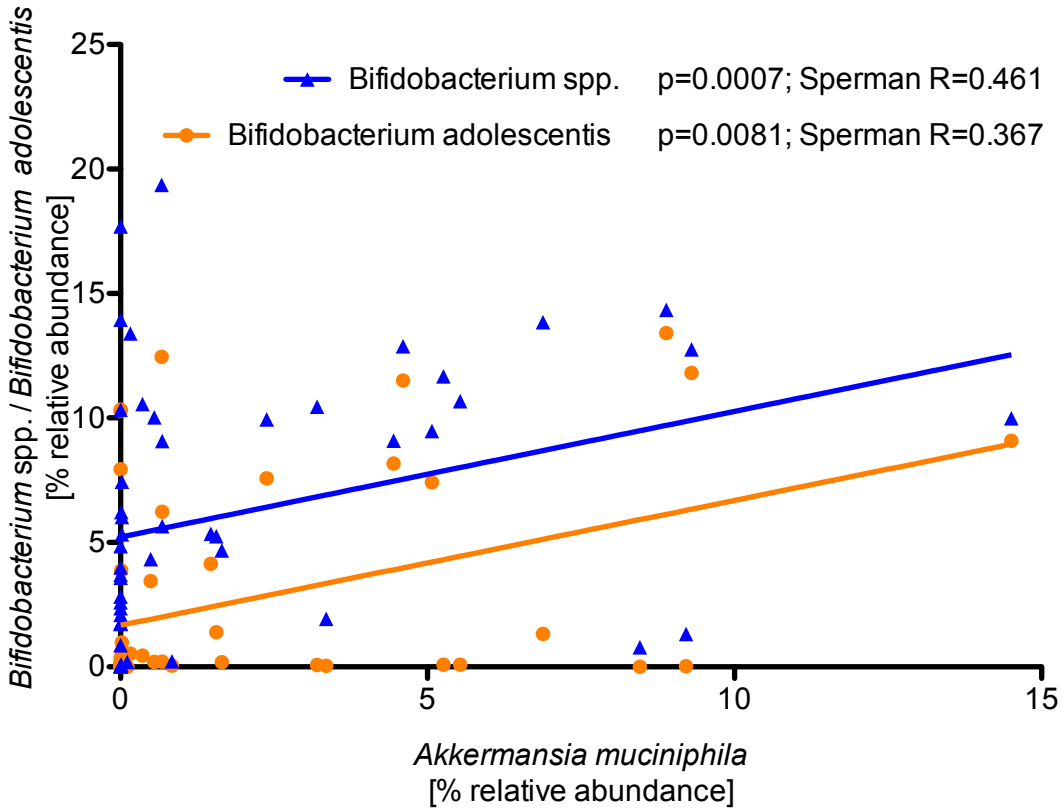


Figure 48. Correlation between *A. muciniphila* and *Bifidobacterium* (*adolescentis*) in donor stool microbiota. Correlation was tested in all donor stool samples regardless of clinical response. Blue triangles indicate rel. abundance of *A. muciniphila* and *Bifidobacterium* spp. for each sample and orange circles indicate rel. abundance of *A. muciniphila* and *Bifidobacterium adolescentis.* Both *Bifidobacterium* spp. on genus level (p=0.0007; Spearman R=0.461) and *Bifidobacterium adolescentis* (p=0.0081; Spearman R=0.367) show significant correlation with *A. muciniphila* according to nonparametric Spearman correlation test (Correlation was tested for n=51).

The same significant correlation could be shown for rel. abundance values of *Ruminococcus* spp. that is also associated with RE donor stool samples, and *A. muciniphila* using a spearman correlation test (Figure 49). On family level there was only borderline significance for correlation between *Ruminococcaceae* and *A. muciniphila* (p=0.0769, R=0.25, data not shown).

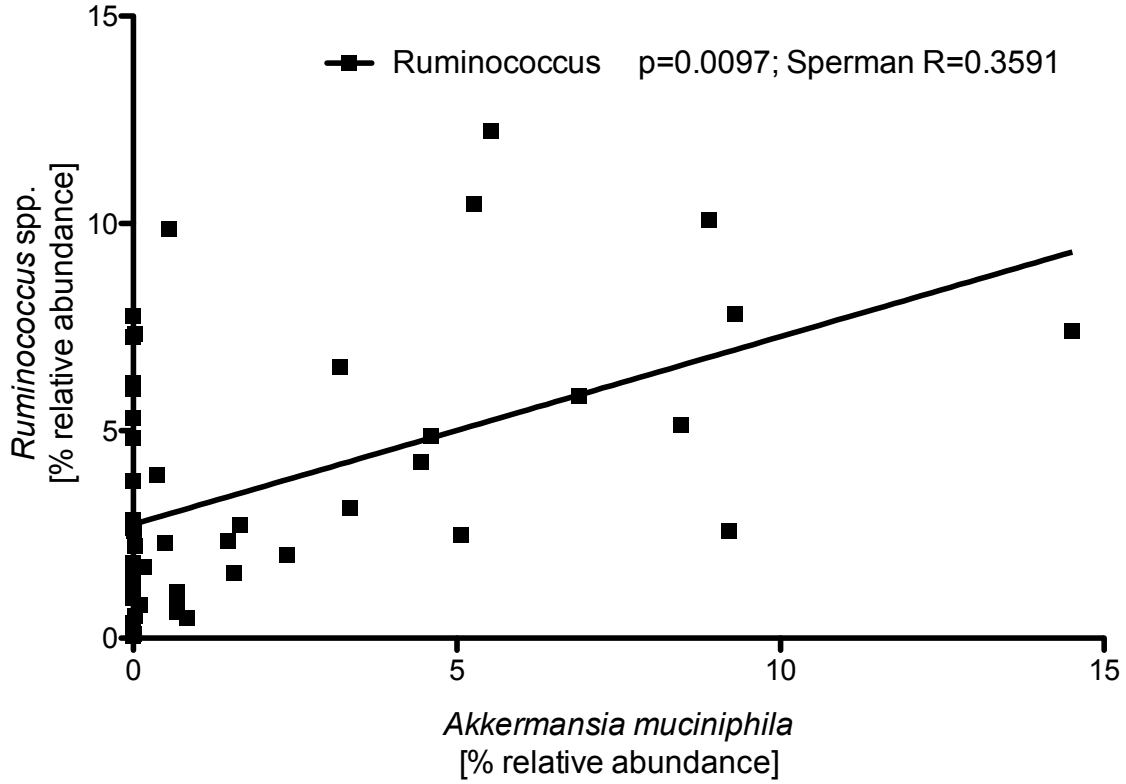


Figure 49. Correlation between *A. muciniphila* and *Ruminococcus* spp. in donor stool microbiota. Correlation was tested in all donor stool samples regardless of clinical response. Black squares indicate rel. abundance of *A. muciniphila* and *Ruminococcus* spp. for each sample. *Ruminococcus* spp. shows a significant correlation with *A. muciniphila* according to nonparametric Spearman correlation test (p=0.0097; Spearman R=0.3591; correlation was tested for n=51).

Interestingly, the success of FMT engraftment in patients did not correlate with clinical efficacy of FMT therapy in patients. Regardless of clinical outcome FMT engrafted to a similar extend in all groups. We compared the unweighted UniFrac distance between recipients and their respective donors at all time points after the FMTs. In all groups, non-responders, partial responders and patients going into remission we saw a significant decrease of the unweighted Unifrac distance from day 3 on compared to day 1. A lower distance value represents a higher similarity between the recipient and the donor microbiota after the first FMT and indicates efficient engraftment in all patient groups. With this comparison we do not see a significant difference of the engraftment efficiency between the different groups according to clinical outcome (Figure 50) (2).

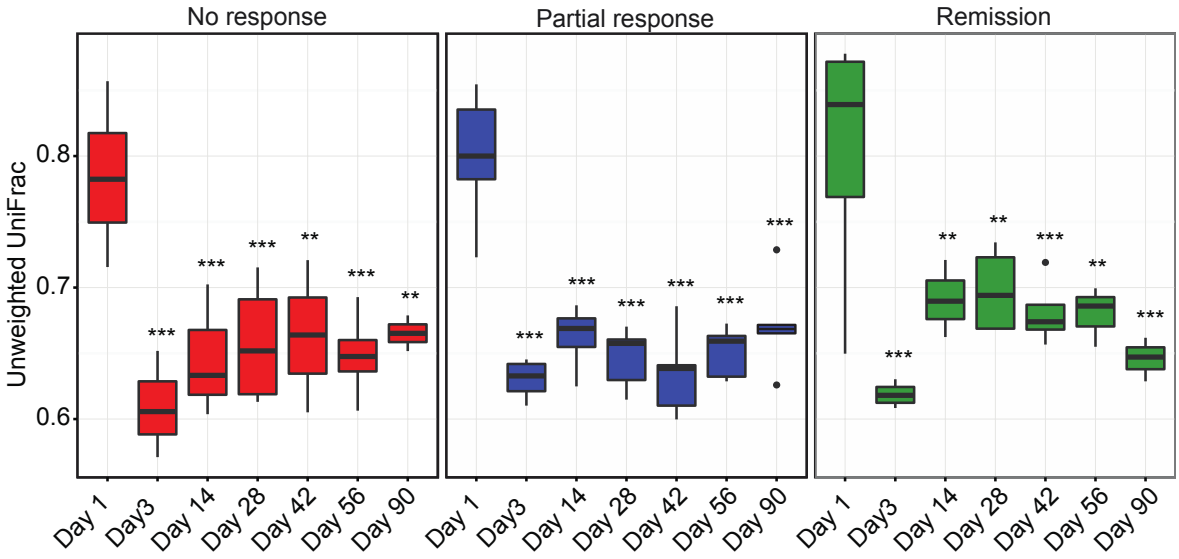


Figure 50. Engraftment of the donor microbiota is unrelated to treatment response using repeated FMTs. A significant and continuous decline of UniFrac distance in all response groups when donor stools were compared to recipients indicates engraftment in all groups. A lower distance indicates a higher similarity of the microbial community composition comparing donors and recipients (red: no response, NR; blue: partial response, PR; green: remission, RE; n=2-5; Two-way ANOVA, Bonferroni posttest **p<0.01, ***p<0.001). Adopted from (2).

The evaluation of the donor microbiota in summary shows that there are clear differences in the taxonomic signature, like increased relative abundance of *A. muciniphila* and *Ruminococcacea*, but also increased microbial richness between donor stool samples that lead to remission and those which lead to no response in UC patients (2).

4.3.1. Effect of antibiotic pre-treatment on the fecal microbiota in UC patients

The 10-day antibiotic pre-treatment already resulted in a decreased total Mayo score by 2 points in all 27 patients at day 1. 5 from the 27 patients even achieved a clinical response after 10 days with the antibiotic treatment alone. However, as stated earlier 5 patients from the antibiotic control group had to be excluded from the study as three of them experienced a *C. difficile* infection after 14 days, one suffered from antibiotic associated diarrhea without pathogen detection and in one patient UC aggravated during antibiotic therapy. In contrast, all 17 patients of the FMT-group completed the study (until day 90), which proves the clinical efficacy

and necessity of the FMTs post-AB treatment. Nevertheless we wanted to see the impact of antibiotics on the microbiota in the short and long run (2).

Richness (observed species) dramatically dropped in stools of all patients after 10-day antibiotic treatment (Figure 51A). Reduced richness could be reversed to a certain extent by FMTs, but in contrast to patients receiving FMTs, those patients from the AB control group receiving no FMTs showed no significant increase in species richness at day 90 compared to samples at day 1 directly after 10 days antibiotic treatment (AB-group, Figure 51B). The fact that the richness remains low in patients without FMT after antibiotic treatment might explain the high dropout rates within this group. A decreased microbial richness and the accompanying impaired colonization resistance is a major risk factor for infections and therefore might be causal for the dropouts due to *C. difficile* infection and antibiotic-associated diarrhea (2).

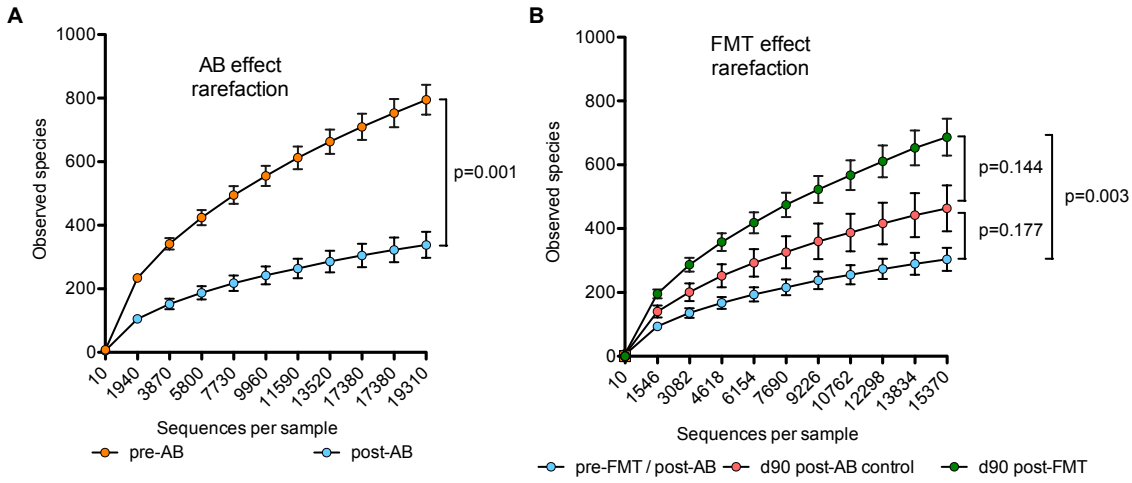


Figure 51. Influence of antibiotic pretreatment on species richness. A: Richness significantly drops after antibiotic (AB) treatment (orange: pre-AB; light-blue: post-AB; n=17-25; p-value = 0.001; mean ± SEM,19315 reads/sample; two-sample t-test.) B: FMT significantly increased species richness in contrast to controls treated with antibiotics alone on day 90 compared to day 1 (post antibiotic therapy). Alpha diversity (observed species); day 1 after antibiotic pre-treatment, day 90 antibiotic control group, day 90 FMT-group (light-blue: pre-FMT/post-AB, day 1; pink: day 90 post-AB control; green: day 90 post-FMT; n=5-25; mean ± SEM 15376 reads/ sample; two- sample t-test). Adopted from (2).

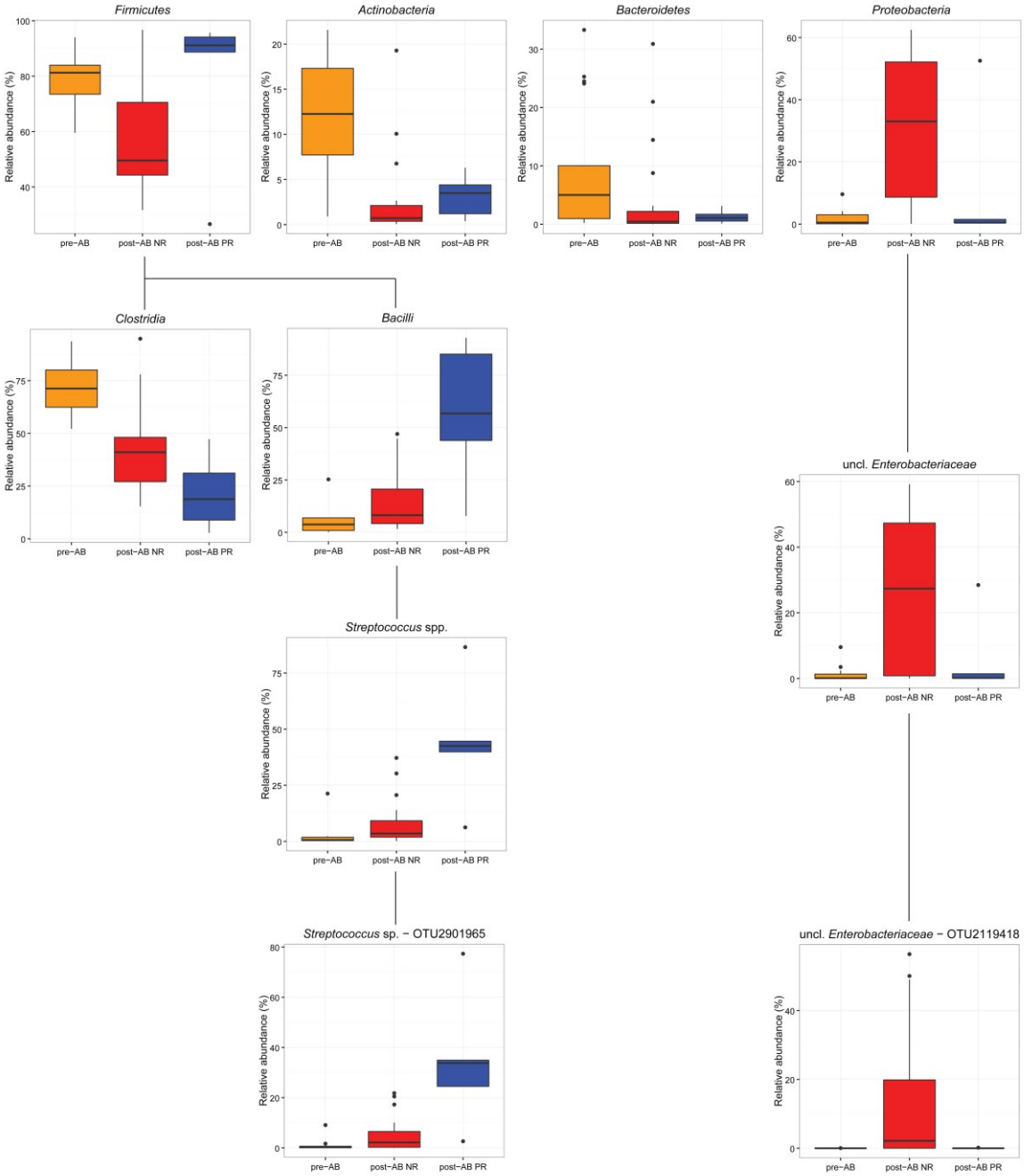


Figure 52. Fecal microbiome composition after antibiotic pretreatment according to the response of patients to antibiotics (AB). Comparing patient stool samples at day -10 pre-AB treatment and on day 1 post-AB treatment; 5 patients that showed a PR and 20 patients show NR. (Orange: pre-AB; red: post-AB NR; blue: post-AB PR; n=5-20; significantly different taxa are indicated with LDA- Score (>4.6), p-value (<0.05) and associated category according to LEfSe). Adopted from (2).

As there were 5 patients in the overall study achieving signs of clinical response at day 1 only due to antibiotic pre-treatment, we compared the microbiota from these patients at day 1 (post-AB PR) to those who didn't show signs of clinical response at day 1 (post-AB NR) and to baseline microbiota ahead of the antibiotic pre-

treatment (pre-AB). No patient achieved remission at day 1 due to antibiotics. The microbiota composition and the taxonomic profile showed various differences between patient samples. At baseline, patients showed significantly increased levels of *Actinobacteria*, *Clostridia* and *Bacteroidetes* ahead of the antibiotic therapy according to a LEfSe analysis. Whereas patients pre-AB are dominated by *Clostridia* the microbiota of patients achieving a clinical response post-AB (post-AB PR) showed a significant increase of relative abundance of *Bacilli* compared to the other two groups. Especially the relative abundance of *Streptococcus* was significantly increased in post-AB PR samples. On the contrary the microbiota of all other patient samples that show no clinical response due to antibiotic treatment on day 1 (post-AB NR) showed a significant increase of *Proteobacteria* and *Enterobacteriaceae* (Figure 52) (2).

The antibiotic therapy of course has a strong impact on the microbiota of patients with chronic active UC. In some patients it even leads to a short term clinical response that is associated with high levels of *Streptococcus* and low levels of unclas. *Enterobacteriaceae.* Nevertheless reduced microbial diversity persisted in patients after antibiotic treatment without receiving FMTs (2).

4.4. Discussion

FMT has been shown to be a highly efficient method to treat recurrent *Clostridium difficile* infection (107). Although FMTs have been studied in small randomized controlled trials (113–116) and various case studies (112,216–221) as treatment of UC, the results are up to now controversial for UC due to varying response rates to FMT. The results of this study suggest that the donor's microbiota composition may play a crucial role for an efficient FMT and leading to treatment response in patients with therapy refractory chronic UC. Among other taxa, higher relative abundance of *A. muciniphila* and *Ruminococcaceae* as well as increased bacterial richness of the fecal microbiota characterizes donor stools leading to a successful therapy compared to donors without efficacy (2).

A recent study by Moayyedi et al. supports the importance of a specific donor microbiota as a factor for successful FMT treatment in UC (113). Although they did not associate a specific donor microbiota with remission, this study showed that success of therapy was restricted to certain donors. In a later study the authors could show in a small case series with IBD patients, treated with FMT, that response in patients was associated with higher bacterial richness (185) corresponding to our findings (2).

This study further suggests that taxonomic composition in the donors' microbiota with presence of high levels of *Ruminococcacea* in general and *A. muciniphila* in particular, and absence of certain bacteria is crucial for the success of FMT treatment in therapy refractory UC patients. *A. muciniphila* generated a great scientific interest since its discovery by Derrien et al. as a human intestinal mucin-degrading bacterium. Being the only known representative member of its phylum *Verrucomicrobia* it harbors many genes that are unique for the human microbiome (222). Interestingly *A. muciniphila* is inversely associated with metabolic disorders such as obesity, T2D, dyslipidemia, cardio metabolic diseases and low-grade inflammation (223,224). It could already been shown that the relative abundance of 16S rRNA gene copies from *A. muciniphila* are reduced in colonic mucosal biopsies from inflamed CD as well as inflamed and non-inflamed UC patients compared to healthy controls (213). Additionally it could be shown that *A.*

muciniphila, together with other anaerobic taxa, play an important part in mucosal wound-healing in mice. *A. muciniphila* also enhances wound restitution in a *Fpr1*-dependent manner, with similar effects in a dextran sodium sulfate (DSS) induced colitis mouse model (225). Interestingly we could identify a short-term colonization determined by an increased relative abundance of *A. muciniphila* in patients, achieving clinical signs of remission at the end point of the study, 3 days after the first FMT. Long-term colonization however was not essential for clinical response in patients after FMT as there was no significant increase of *A. muciniphila* in any patient after 14 days or later. One reason might be that already a short term colonization or simply the transfer of a transplant, enriched with *A. muciniphila,* provides anti-inflammatory capacities. It could already be shown for *A. muciniphila* that already a treatment with bacterial outer-membrane vesicles (OMVs), which represent the antigenic structures of the outer-membrane molecules and allow translocation of signal molecules to other bacteria or host cells, is protective against the progression of DSS colitis in mice (226). It is also possible that *A. muciniphila* is simply a marker for a healthy and beneficial donor microbiota or is required for subsequent recolonization of the UC patients colonic mucosa by other essential commensals similar to sequential colonization process of *B. thetaiotaomicron* and *F. prausnitzii* in germ-free rats (194). Despite the great potential of the commensal bacterium, its positive effects are discussed quite controversial in general. *A. muciniphila* was recently also associated with Parkinson's disease (PD) (227,228), MS (229,230), Alzheimer's disease (AD) (231) and promoting intestinal inflammation in an IL-10 knockout colitis mouse model (232). Thus the role of *A. muciniphila* associated with FMT in chronic active UC needs further investigations such as double-blinded controlled clinical trials where a single donor is used for multiple patients. This study setup would further elucidate on the treatment efficacy of a certain donor microbiota signature. The other taxa in donors microbiota from our study that were associated with remission in UC patients were unclassified *Ruminococcaceae* as well as *Ruminococcus* spp. Noteworthy the discovered OTUs in this group only show little homology to *R. gnavus* and *R. torques*, which have been shown to be increased in UC patients (213) and in donor microbiota that doesn't cause response in UC patients (214). Instead they show close similarity to *R. bromii* and *R. champanellensis* (Figure 45). Interestingly these bacteria have been demonstrated to be able to process

non-digestible resistant starch fibers and thereby promoting the production of SCFAs (233–235). This could also explain the improvement of patients, as it is known that the SCFAs acetate, butyrate and propionate are dramatically decreased in fecal samples in IBD (89,100) IBD has already been associated with altered bacterial fermentative pathways that influences SCFA production (208,236). Early treatments with SCFA could already show its clinical effectiveness by amelioration of colitis in UC patients (237). It also needs to be mentioned that *F. prausnitzii*, which has ant-inflammatory capacities and was reported to be decreased in IBD (208,238), was not increased in the donors that caused remission in patients. Other taxa that were found to be increased in the microbiota from the same family *Ruminococcaceae* are maybe able to substitute *F. prausnitzii* in the microbiota of UC patients and complement its anti-inflammatory capacity. The large variations of response rates to FMT treatment from 0-100% in UC patients in various studies (112–116,216–221), including the inconsistent results of the four randomized controlled trials (113–116), could be an indication for the significance of a specific signature in the donor microbiota. The protocol of the study from Paramsothy et al. was based on a mixed FMT from multiple donors. This procedure increases the probability of transferring a beneficial microbiota to UC patients which might explain the better outcome in general over the placebo group in this trial (115).

Dialister sp. was significantly reduced by antibiotic therapy and the relative abundance also remained significantly decreased in patients achieving remission at the end of FMT treatment, compared to partial and non-responders (2). This could indicate towards an altered recolonization of the colonic mucosa in patients achieving remission. Interestingly increased levels of *Dialister* sp. were noted in IBD and in spondyloarthritis, in the latter case patients often show microscopic signs of chronic bowel inflammation resembling early CD (239). Nevertheless the importance of this finding remains to be further investigated using metagenomic approaches with better resolution as the similar levels of *Dialister* sp. could be detected in all donor groups and may be species or strain specific. In contrast to the findings from other studies we could not determine differences between patients achieving remission and non-responders regarding microbial diversity or the overall engraftment of the transplant between response groups (114,115,214).

The heterogeneity of the patient population and the applied FMT protocol might also affect the efficacy of the treatment. We identified male sex and an increased total Mayo score before FMT as predicting factors for a positive treatment response. Other studies showed that a positive treatment response was associated with an overall shorter disease duration as well as ongoing immunosuppressive therapy (113). We used antibiotic pre-treatment to reduce the endogenous, potentially harmful microbiota and to overcome intestinal colonization resistance.. After 10 days of antibiotic pre-treatment a significant part of UC patients already showed a partial response defined as reduction of the total Mayo score by at least 3 points. In regard to taxonomic composition these partial responders showed significantly decreased relative abundances of *Enterobacteriaceae*, which are known to be pro-inflammatory, generally showing increased concentrations during dysbiosis (240). Furthermore these partial responders showed significantly increased levels of *Streptococcus* spp., with close similarity to *S. thermophilus*. This species is commonly used as probiotic, it could also be shown to reduce clinical signs of colitis in a DDS- colitis mouse model (241) and it has beneficial effects when used in pouchitis (242). Despite the clinical response to antibiotics for a small subpopulation, the control-group in general showed worse course and was poorly tolerated. 50% of the antibiotic treatment control group showed severe side effects such as antibiotic-associated diarrhea, *C. difficile* infections and exacerbation of UC. These results add further knowledge to the current understanding of antibiotics used in UC and its varying effects (210). The high rate of side effects in the antibiotic treatment control group might be due to their low microbial richness which could lead to a loss of intestinal colonization resistance and subsequent infections such as with *C. difficile*. In combination with repeated FMTs this antibiotics protocol was remarkably well tolerated in UC patients, as all patients already failed previous biologic and/or immunosuppressive therapies (2).

This study has limitations due to the small number of patients and missing randomization in treatment groups. Nevertheless it provides further evidence for the efficacy of FMT treatment in IBD, especially in therapy refractory UC (2). Further studies need to illuminate the mechanisms of the treatment and the microbial composition of UC patients and suitable donors. Our findings already

indicate that a donor selection process for a certain microbial signature has to be considered for FMT treatment of UC patients in the future.

5. Conclusion

In conclusion the results of the three described projects provide novel and significant data for the field of microbiota research in various fields. This includes a refined characterization of the taxonomic compositions in the human upper GI tract and treatment strategies of diseases signified by dysbiosis via microbiota restoration.

This work provides evidence that the upper GI microbiota is homogenous throughout different locations and is influenced by microbial communities from upstream GI locations. Different sections of the stomach show varying relative abundances of specific bacterial taxa during their passage to the duodenum. The workup of differences in the upper GI tracts microbial communities in humans brought important findings to light. The microbial diversity between patients showed greater variations between subjects than between the different locations of the upper GI tract. Nevertheless significant differences in the taxonomic composition between esophagus and stomach including gradual changes of e.g. *Streptococcus* concentrations within the stomach could be detected. An important finding with potentially far-reaching clinical implications is represented by the variations of the relative abundances of *Firmicutes* and *Bacteroidetes* in the luminal gastric microbiota that were evident independent of clinical implications. Despite the predominant colonization of *H. pylori* in corpus and antrum microbiota of patients with *H. pylori* associated gastritis, we saw that the luminal microbiota in the gastric aspirate of the same patients, only show minimal concentrations of *H.pylori*. Interestingly the relative abundance of *Firmicutes* and *Bacteroidetes* in these samples is within the same range as in the gastric aspirate microbiota from non-infected patients. The results from comparisons of the gastric microbiota between humans and animals, including significant differences of the dominant taxa on genus level (e.g. *Streptococcus* vs *Lactobacillus*), must be considered when conducting animal experiments with mice or gerbils on gastrointestinal infectious diseases, like *H. pylori* associated gastritis. Future studies should increase the number of animals and individuals for a defined disease context, such as autoimmune gastritis. Furthermore they should include longitudinal samples of the same individuals to address the possibilities of diurnal compositional

fluctuations for instance caused by the intervals between food intakes. As this study was based on microbial DNA, future studies should also include RNA based microbiota analysis to identify metabolically active bacteria using metatranscriptomic assays. Additionally DNA shotgun metagenomic sequencing from the upper (saliva, gastric and duodenal aspirate) and lower GI tract (fecal samples) from the same subjects would further improve the resolution of the microbial community. This is important for an absolute quantification and strain-specific identification of microbes that originate from and survive the upper GI tract and have the ability to colonize the lower GI tract despite the harsh changes of environmental factors. This would improve our understanding of microbial dynamics through the human GI tract that helps for future clinical probiotic applications to fight dysbiosis and restore the microbiota.

The second part of the thesis describes a severe form of enterocolitis with a severe apoptotic phenotype in critically ill patients that developed a therapy-refractory high-volume diarrhea after concomitant treatment with antibiotics and corticosteroid steroids in the ICU. A disease which was hitherto not described. Alongside the absence of a defined pathogen for this disease, these patients do not have an established intestinal microbiota, determined by absolute quantification of bacterial DNA in fecal samples, which enables colonization resistance against enteropathogens. The loss of intestinal microbiota leads to strong fluctuations in the taxonomic composition of the patients microbiota, dominated only by few bacterial species, that are generally considered as commensals of the skin or oropharynx microbiota and aren't part of the normal colonic microbiota. The massive dysbiosis could trigger this severe form of apoptotic enterocolitis. These conditions might be relieved by FMT as it leads to a reestablishment of a physiologic gastrointestinal microbiota, that might not spontaneously happen in the ICU, as shown in a single case (1). The data from the single-FMT provides further knowledge to answer the question whether or not FMT should be used in critical ill patients with severe GI complaints (118,243).

The third part of this thesis showed that our established FMT protocol after antibiotic treatment was very well tolerated and also lead to a clinical response, indicated by a reduction of at least 3 points in the total Mayo score at the end of

the therapy, in 59% of the patients. 4 out of 17 patients even achieved clinical remission (total Mayo of 2 or less) (2). This effect of FMT treatment goes in line with other randomized controlled FMT trials in UC (113–116), in this study however the patients showed a persistent illness course with previous failures in immunosuppressive, biologic or both therapies combined. A very prominent finding of this study was that efficacy of FMT treatment in chronic active UC patients was strongly associated with a specific taxonomic composition of the donor's intestinal microbiota including an increased abundance of *A. muciniphila* and *Ruminococcus* spp. amongst others (2). To confirm the efficacy and therapy tolerability of our study protocol, this study needs to be repeated with randomized double-blinded treatment. Furthermore various healthy donors should be recruited to donate for multiple patients and thereby confirm the hypothesis that treatment efficacy depends on the donor microbiota. The microbial signature in the donor microbiota that is associated with treatment success provides important data for future studies including a donor screening for FMTs with a generally increased therapy efficacy. The provided data is also important for the development of specific (anaerobic) microbial preparation such as EcoBiotics that might lead to new straightforward treatments for UC.

6. Bibliography

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