Microbiota profiling in gastrointestinal health and disease

{Special focus on methodological and analytical approaches}

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1

General introduction and outline of the thesis

GENERAL INTRODUCTION

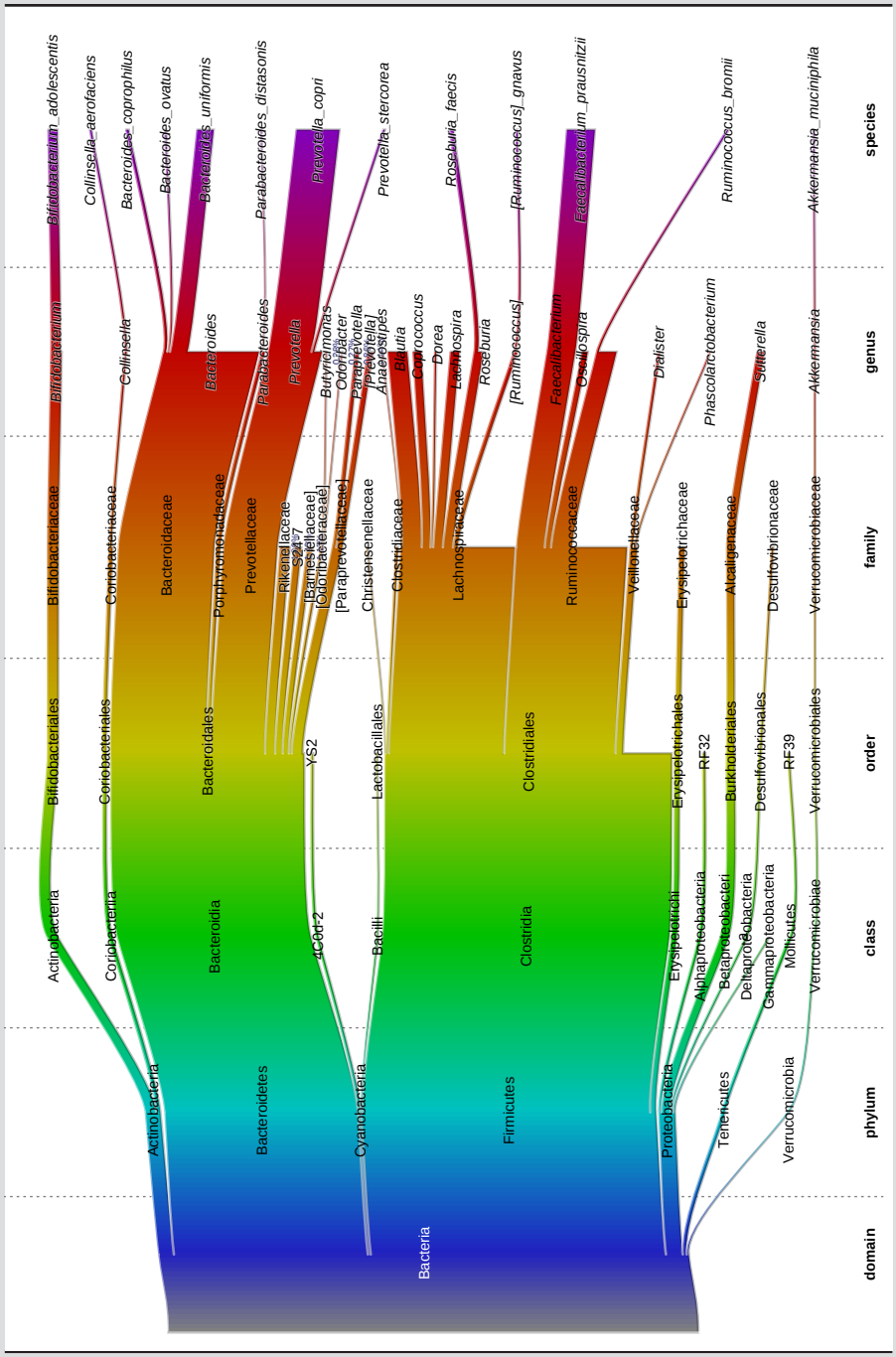
The human body is colonized with at least as many microbes as human cells. Microbes within each of the three domains of life populate the human body, although the bacteria outnumber the archaea and eukaryotes by 2-3 orders of magnitude [1]. The most heavily colonized human organ is the intestine and the collection of these microorganisms is referred to as the intestinal microbiota. The microbiota, referring to the composition, should not be confused with the microbiome which is defined as the collection of genes of all microbes in a community. The human intestinal microbiota is a complex ecosystem in which microbe-microbe and microbe-host interactions contribute to host health. The emerging interest in the role of commensal microbes in health and disease, accompanied by the availability of novel molecular techniques that enable in-depth analyses of large numbers of samples, has led to an explosion of studies on this subject over the past few years.

Composition and shaping of the intestinal microbiota

Although some debate is ongoing, the fetal intestinal tract is considered to be sterile and is colonized by microorganisms during and after delivery. Next to fungi and viruses, the majority of these microorganisms are bacteria. Environmental factors such as mode of delivery, type of feeding and exposure to parents, siblings and the built environment have an effect on the colonization of the newborn intestinal microbiota. The microbiota in newborns is unstable and has a low diversity, and gradually develops into a stable, adult type microbiota during childhood [2]. The composition of the microbiota is also affected by host genetics and impacts maturation and functioning of both the small and large intestine [3]. The adult microbiota composition is rather stable and has a large inter-individual variation, but can be affected by external factors, such as antibiotic use and major dietary changes [4].

The composition and numbers of bacteria increase along the gastro-intestinal (GI) tract, with a low richness and diversity in the upper GI tract, mainly due to the low intragastric pH, and a high richness and diversity in the colon, reaching up to 10^{11} bacteria per ml colonic content [1]. Dominant intestinal phyla are the Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia. Though, their relative abundance and lower taxonomical levels within these phyla (such as Prevotellaceae at family level and *Prevotella* at genus level) vary along the GI tract (see also Figure 1.1 and 1.2), which can be explained by the physiological differences between the compartments of the GI tract. The small intestine for example, has a lower pH, higher oxygen and bile acids levels as compared to the colon, which results in higher numbers of facultative anaerobes, *e.g.* Lactobacillaceae and Enterobacteriaceae, being members of the Firmicutes and Proteobacteria phyla, respectively.

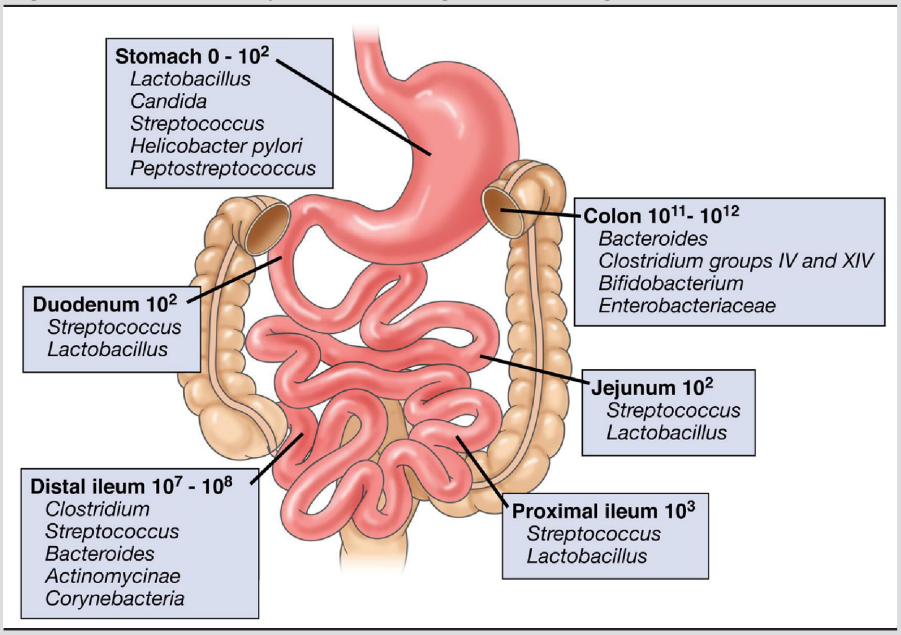
Figure 1.1. Visual representation of the fecal microbiota composition in healthy individuals.



The microbiota is present from the least (domain) to the most specific taxonomic level (species).

The colon, with its relatively low oxygen levels, slow transit, high pH and nutrient density, is mainly colonized by members belonging to the Bacteroidetes or Firmicutes phyla that are able to reside by fermentation of complex polysaccharides, for example Bacteroidaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae and Ruminococcaceae. [5]. Although very few bacterial species are shared between (un)related individuals, microbial functions are found to be rather conserved, as demonstrated by the existence of a functional core by the European Metahit consortium, pointing to the importance of microbiota function in addition to microbiota members identity [6].

Figure 1.2. Distribution of key microbial taxa at genus level in the gastrointestinal tract.



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The function of the intestinal microbiota and its importance for human health

The intestinal microbiota exerts several important functions for host's health, including *e.g.* influencing the intestinal epithelium function and integrity, stimulating the immune system, protection against invading pathogens and its large metabolic activity [7,8]. Animal models showed for example that the absence of an intestinal microbiota was found amongst others to result in an underdeveloped GI-tract and immune system, and a higher susceptibility to infections. Furthermore, vitamin K and B deficiency and a reduced caloric intake was observed [9]. The microbiota protects the host against potential pathogens, *i.e.* contributing to colonization

resistance, by producing antimicrobial molecules, stimulating the production of antimicrobial compounds by the host and by competitive exclusion. The latter occurs by occupation of attachment sites and consumption of nutrient sources [10].

The microbiota is also vital for the maturation and functioning of the intestinal epithelium. This comprises for example cell proliferation and differentiation, enzyme and hormone secretion and the structural integrity and functioning of the intestinal barrier, by impacting *e.g.* mucus secretion and the junctional complex sealing epithelial cells. For example, *Bacteroides thetaiotaomicron* is able to induce sprr2A, a protein that is necessary for the maintenance of desmosomes at the epithelial villus, and bacterial peptidoglycan maintains the tight junctions via TLR2 mediated signaling [11,12]. Other bacteria however, can damage the intestinal barrier and thereby promote inflammation. For example, adherent invasive *Escherichia coli* (AIEC) is able to adhere to invade the epithelial cells by recruiting the host cell actin and microtubule, thereby leading to increased permeation of bacteria and their products [13,14].

The microbiota is also able to modulate the immune system by producing signaling metabolites and interacting with epithelial and immune cells. For example, *Bifidobacterium breve* is able to dampen the inflammatory response of B cells by reducing inflammatory cytokines [15]. The production of interleukine 10 (IL-10) by regulatory T cells can be induced by polysaccharide A of *Bacteroides fragilis* and short chain fatty acids (SCFA) resulting from bacterial fermentation of (complex) carbohydrates [16,17]. Moreover, segmented filamentous bacteria (SFB) stimulate the development of T helper 17 cells [5].

Finally, the microbiota can be characterized by its large metabolic activity, playing an important role in host metabolism. Members of the microbiota are for example responsible for the synthesis of certain vitamins, which cannot be synthesized by the host (*e.g.* vitamin K and vitamin B). Furthermore, the microbiota has the potential to increase energy harvest from the host diet in the form of SCFA as a result of saccharolytic fermentation. SCFA exert many beneficial effects on the intestinal health as they are an important energy source for epithelial cells, and have *e.g.* anti-inflammatory and anti-oxidative effects, improve barrier function and reduce satiety [18]. On the other hand, proteolytic fermentation by the microbiota results in the production of potential harmful metabolites such as ammonia, indoles and phenoles [19]. Moreover, certain members of microbiota are able to metabolize drugs, thereby affecting their effectivity [20].

As the microbiota plays an important role in maintaining intestinal homeostasis, microbial perturbations can have detrimental effects on the homeostasis of the host and thereby contribute to various disorders. Shifts in the microbiota composition have been associated with both intestinal (*e.g.* inflammatory bowel diseases (IBD)) and extra-intestinal disorders, including for example metabolic disorders (*e.g.* obesity) and chronic liver diseases (*e.g.* liver cirrhosis).

Intestinal disorders

First interest on the contribution of the intestinal microbiota in health and disease focused on intestinal disorders, including for example Irritable bowel syndrome (IBS) and Inflammatory Bowel Diseases (IBD). IBS is a functional disorder, characterized by abdominal pain and altered bowel habits with a prevalence up to 11% in the general population [21]. In addition to an altered motility, permeability, visceral hypersensitivity and low-grade intestinal inflammation, an altered intestinal microbiota composition and activity have been demonstrated in patients with IBS [22].

IBD is perhaps the most investigated disease associated with the microbiota. It is a chronic relapsing intestinal inflammatory disease, and comprises ulcerative colitis (UC) and Crohn's disease (CD). IBD is associated with clearly impaired quality of life and high health care costs [23,24]. The incidence of UC and CD have been rising over the past decades in both developed and developing countries, which will further impact both patients as well as society [25].
While inflammation in UC is mainly superficial and restricted to the colon, CD is characterized by transmural and discontinuous inflammation, which can involve the entire GI tract [26]. In periods of active inflammation, CD is associated with symptoms such as abdominal pain and (bloody) diarrhea [23,26]. The representation of the disease is heterogeneous and the disease course is poorly predictable. There is currently no cure for this disease and treatment focuses primarily on reducing symptoms. However recently, obtaining mucosal healing (with absence of ulcerations and erosions) is becoming more important as treatment goal [27]. Monitoring the mucosal inflammation is crucial to prevent disease progression and complications, but the current golden standard (endoscopy) is an expensive and invasive procedure with risk of complications. Clinical activity scores and general inflammatory markers do not correlate well with mucosal inflammation. Currently, the identification and validation of novel biomarkers is therefore of great interest within the CD field.

The exact cause of CD is not known, but it has generally been accepted that CD is the result of an incorrect immune response against the commensal microbiota in genetically predisposed individuals [28]. Early studies focused on identifying one or several key players that could induce intestinal inflammation, *e.g. Mycobacterium avium* and a number of Proteobacteria, including *Campylobacter* and AIEC [29]. As findings were not conclusive, the focus has shifted towards the microbiota as a whole. Several consistent observations have been reported for CD when compared to health controls: *i.e.* a reduced microbial diversity, reduced levels of Firmicutes (in particular *F. prausnitzii)* and an increased abundance of Enterobacteriaceae *(*in particular *E. coli*) [30–36].

Studies on treatment with antibiotics and washing out the gut lumen, indicated that the intestinal microbiota might also be associated with the development of exacerbations [37,38].

Differences between CD patients in remission and during exacerbations have been reported, but results are not consistent [36,39–47]. Inconsistencies can be explained for example by differences in methodology (*e.g.* definition of active disease) used to examine the microbiota and between patients included (*e.g.* differences in disease location and medication use). In addition, the majority of the studies conducted so far did not investigate the microbiota within patients with changing disease activity over time, which is crucial considering the large inter-individual variation of the microbiota.

Obesity

In the last decade, studies on the role of the intestinal microbiota in obesity are emerging. For adults, a body mass index (BMI) of ≥30 kg/m^2 and a BMI ≥40 kg/m^2 is defined as obese and morbid obese, respectively [48]. Obesity is associated with low-grade inflammation, decreased intestinal barrier function and co-morbidities such as cardiovascular disease, type 2 diabetes and non-alcoholic fatty liver disease, and has a substantial impact on direct healthcare as well as indirect costs (*e.g*. loss of work productivity). The prevalence of obesity is rising in both developed and developing countries, particularly in urban settings, and is currently linked to more deaths worldwide than underweight [48,49]. A western lifestyle characterized by an increased intake of energy-dense food and a decrease of physical activity are the major causes of the worldwide obesity epidemic. However, recent evidence is accumulating that also the intestinal microbiota is involved in the etiology of obesity by increasing host' energy harvesting efficiency via the production of SCFA and by promoting fat storage via gene expression of *e.g. ANGPTL4*. The first study demonstrating a relation between the intestinal microbiota and obesity reported an increased Firmicutes/Bacteroidetes (F/B) ratio in genetically obese as compared to lean mice [50]. This has been confirmed in a human study, in which a decrease of this ratio was observed in obese individuals after a weight loss program [51]. Although others have confirmed an increased F/B ratio in obese as compared to lean individuals, this ratio remains controversial since some studies reported no or an opposite trend [52]. The observed differences at the phyla level are difficult to interpret since the Firmicutes and Bacteroidetes phyla consists of more than 250 and 20 genera, respectively, and their role in obesity, especially in relation to weight loss, has not yet been clarified. Furthermore, a lower diversity and a higher level of Actinobacteria and reduction of Verrucomicrobia and *Faecalibacterium prausnitzii* has also been reported in obese individuals as compared to lean individuals [53].

The treatment of obesity is challenging, as simple interventions such as caloric restriction and exercise are often not effective. Bariatric surgery is recognized as the most effective long-term treatment for morbid obesity and its co-morbidities. The Roux-en-Y gastric bypass (RYGB) and sleeve gastrectomy (SG) are the most common performed bariatric procedure with good long-term outcome [54,55]. The RYGB procedure results in a small gastric pouch and bypasses the distal stomach, duodenum and proximal jejunum. An increase of richness, facultative anaer-

obes and bacterial taxa common in the upper gastrointestinal tract has been reported after the RYGB procedure, which in part may be caused by the anatomical changes of the GI tract in addition to the weight loss per se. Whether these changes are different after the less invasive SG procedure is unclear. In the SG procedure, weight loss is achieved by resecting the fundal part of the stomach, thereby reducing the stomach size and leaving the intestinal tract intact. SG is increasingly applied as it is a rather simple and safe procedure [56]. Despite its increasing popularity, only one previous study has investigated the microbiota composition of obese individuals after SG [57].

Liver cirrhosis

Cirrhosis is the advanced stage of liver fibrosis, a process in which injured liver tissue is encapsulated or replaced with collagenous scar tissue, as a result from chronic liver diseases [58]. According to a recent study, liver cirrhosis has an incidence of 14-26 cases per 100,000 persons and is the cause for 170,000 deaths per year in Europe alone [59]. Disease progression is characterized by an asymptomatic stage of compensated followed by a symptomatic stage of decompensated disease. The latter is characterized by the development of clinically evident complications of ascites, variceal hemorrhage, hepatic encephalopathy and/or jaundice [60]. The Child-Pugh classification is often used in the clinic to assess the severity of liver cirrhosis. By assigning a score (ranging from 1-3) for five different clinical features (serum levels of bilirubin and albumin, ascites, hepatic encephalopathy and prothrombin time or prolongation for international normalized ratio), patients are classified as class A (best), B (moderate) or C (worse) [61]. At present, the only curative treatment is liver transplantation [58]. Therefore, further insight in factors contributing to the development and progression of liver cirrhosis is highly warranted.

The most common causes of cirrhosis are chronic liver disease related to alcohol consumption (alcoholic liver disease), hepatitis virus infections and (morbid) obesity (leading to non-alcoholic fatty liver disease). Furthermore, emerging evidence suggests that increased intestinal permeability contributes to the pathogenesis of several liver diseases. The liver is continuously exposed to intestine-derived molecules, including nutrients as well as bacterial components (*e.g.* peptidoglycans and endotoxins), by its blood supply from the portal vein. Hepatic cells, including Kupffer cells and sinusoidal cells, respond to the microbial influx via pathogen recognition receptors (PRR) [29]. Non-alcoholic fatty liver disease and alcoholic liver disease have been reported to be associated with increased intestinal permeability and an altered composition of the intestinal microbiota [62]. Currently, also a relation between the intestinal microbiota and liver cirrhosis is increasingly recognized. For example, dysfunction of the intestinal epithelial barrier in patients with liver cirrhosis patients enhances bacterial translocation, which induces inflammation in the liver and thereby aggravates liver injury [63]. Furthermore, small intestinal bacterial overgrowth (SIBO) has been reported in patients with liver cirrhosis. Recent studies have shown that the microbiota composition is not only different between liver cirrhosis

patients and healthy individuals, but also between liver cirrhosis patients with different stages of disease progression. An increase of Proteobacteria and Fusobacteria and a decrease of Bacteroidetes has been observed in liver cirrhosis patients as compared to healthy individuals [64–66]. Alterations in the microbiota were more pronounced in decompensated liver cirrhosis than in compensated liver cirrhosis [67,68]. The number of studies is however still limited and based on complex analyses. Moreover, data on the mucosa-associated microbiota are hardly available and the relevance for daily clinical practice is not yet clear.

As the host depends on the intestinal microbiota for vital functions, the intestinal microbiota contributes to health and may influence disease onset and progression. Consequently, the microbiota can be used to unravel underlying mechanisms to gain insight into the pathophysiology of diseases. In the future it may be even possible to use intestinal microbiota perturbations as biomarkers for health, specific diseases or disease progression [69]. However, investigating the intestinal microbiota is complicated by its large myriad of microorganisms with different functions, the inter-individual variation, potential confounders such as medication use and practical issues with regard to representative biosample collection.

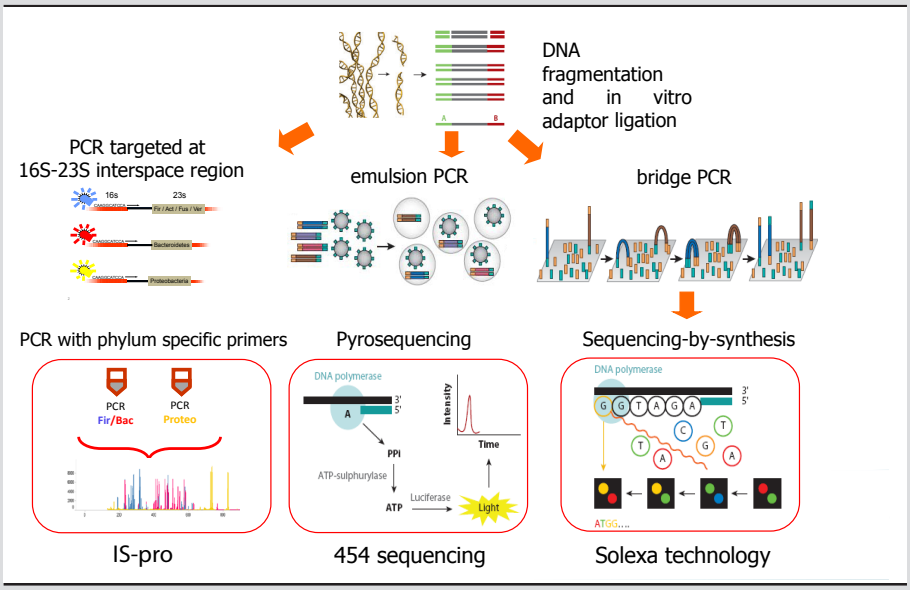
Investigating the microbiota by culture-dependent and culture-independent techniques
First studies investigating the intestinal microbiota composition used culture techniques based on media selective for specific microbial groups, and subsequent biochemical identification of relevant colonies. The arrival of matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) enabled the rapid identification of large numbers of bacterial colonies, thereby significantly reducing the workload [70]. These techniques are reproducible, but cannot capture the complexity of the total microbial community as the majority (>80%) are strict anaerobes and/or cannot be cultured (Eckburg et al, 2005).

Recently, molecular-based techniques to study both the composition and functional capacity of the intestinal microbiota are emerging. The most commonly targeted bacterial gene for taxonomic identification is the 16S ribosomal RNA (rRNA) gene. The 16S rRNA gene is small (1.5 Kb in size) and is highly conserved with nine hypervariable regions that can be used for the identification of bacterial taxa. These nine variable regions (V1-V9) are flanked with conserved regions, which can be used as targets for PCR [71]. The first assessment of the intestinal microbiota of three healthy individuals was published by Eckburg *et al.* [72]. The group used a full-length 16S rRNA sequencing method, also known as Sanger sequencing, to assess the microbiota composition. This method has a high taxonomic resolution and sensitivity, but is limited by the high costs.

Currently, more cost-effective so-called next-generation sequencing (NGS) techniques have replaced the Sanger method. With these next-generation sequencing techniques only a

selected part of one or more hypervariable regions of the 16S gene are being sequenced. The main advantage is the speed and high-throughput capacity. The reduced time and manpower needed for sequencing result in a lower cost, making these techniques available for a wider audience. The 454 pyrosequencing technology of Roche was one of the first NGS techniques used, but has difficulty distinguishing sequences of identical bases (homopolymer errors). The Illumina MiSeq system was released later and outperforms the 454 system in terms of data quality and costs. Currently, the Illumina MiSeq system is the preferred choice for 16S rRNA gene sequencing [73]. A visual representation of the 454 and Illumina MiSeq system is depicted in Figure 1.3.

Figure 1.3. Comparison between different techniques that are used in this thesis to assess the microbiota



Although the number of studies in the intestinal microbiota is exploding, comparisons between studies are hindered by differences in methods used. Primer selection is important since it can influence the observed abundance estimations of the microbiota [73–75]. For example, the V4-V6 and V7-V9 region fail to detect the genus *Fusobacterium* and *Selenomonas,* respectively [75]. Furthermore, variability across studies and errors can be caused by sampling methods, DNA isolation procedures, library preparation (amplification, purification, quantification) and sequencing depth [76–78]. To determine the microbiota composition and diversity, sequences are binned into Operational Taxonomic Units (OTUs) based on their percentage sequence identity. Certain thresholds (90%, 95%, 97% and 99%) are accepted as indicators of taxonomic rank

(family, genus, species and strain, respectively) [4]. The analysis of these data is complex and advanced knowledge of biostatistics is needed, making this procedure not readily applicable for the clinic. Therefore, use of other techniques should be explored. IS-pro (16S-23S interspacer region-based profiling method) is a validated PCR based bacterial profiling technique, which combines phylum classification by phylum-specific fluorescent-labeled primers and subsequent species differentiation by the 16S-23S rRNA intergenic spacer region (see also figure 1.3). This technique is easy to implement in clinical practice enabling analyses of single samples and does not require extensive specialized bioinformatic knowledge. This technique is however not as widely applied as next-generation sequencing and therefore needs further validation.

Human microbiota studies: sample collection, study designs and confounders
In addition to technical aspects of the molecular microbial methods being used, analyses of the microbiota will be affected by the type of biosample analyzed and the procedure for collection and storage thereof.

The human microbiome project (HMP) has investigated the microbiota of different body sites and while some of these are relatively easy to access, such as the skin and oral cavity, sampling of the gastrointestinal tract remains a challenge [79,80]. Current studies on the intestinal microbiota mainly focus on fecal samples and to a lesser extent on mucosal biopsies, presumably due to invasive procedures needed for obtaining mucosal biopsies. The fecal microbiota does however not represent the entire gastrointestinal tract and conclusions obtained from studies using feces as a proxy for the microbiota of the gastrointestinal tract need to be interpreted with caution [72,81]. However, as fecal samples are relatively easy to obtain, they facilitate analyses in large cohorts as well as follow-up studies.

Different sampling and storage methods can also be an important reason for variability between different studies, as temperature can cause shifts in the microbiota community and DNA degradation may occur because of oxidation, hydrolysis and enzymatic degradation. Still, sampling and storage methods are often not standardized and addressed only briefly in methods sections. Fecal samples should ideally be frozen directly before transfer to -80°C, but this is not always feasible when studying various groups of subjects from daily clinical practice. Sampling and storage methods need to be patient-friendly, clinical implementable and should have minimal effect on the microbiota composition.

Also choices regarding the study design are crucial in human microbiota studies to ensure correct and unbiased results. Variables that potentially have an effect on the microbiota composition should be balanced between experimental groups, but selection of study participants may be limited by the population that is available for the study. Case-matched controls are preferred, with use of the same methods for sample collection and molecular analyses.

Common factors that can influence the microbiota, such as diet, BMI, age, pregnancy, ethnicity, stool consistency and medication use, should also be taken into account [82,83]. Medication use, and in particular antibiotics, have a profound and medium to long-term effect on the microbiota. In addition, each antibiotic can have a different effect on the microbiota and the antibiotic response can vary between individuals [83,84]. Furthermore, cross-sectional studies are hampered by the large inter-individual variation. This can (in part) be overcome by conducting longitudinal studies, where subjects can be their own control. Longitudinal studies can provide information about the microbial changes over time and resilience after interventions, which are previously shown to be altered in various diseases or preclinical states [83].

AIMS AND OUTLINE OF THE THESIS

A large body of evidence supports the role of the intestinal microbiota in the development and/or progression of various diseases. Still, the impact for clinical practice is often not clear and findings can be affected by sampling methods and study designs, as well as by the applied molecular tools and statistical analyses.

In the present thesis, we aimed to get further insight into the role of the intestinal microbiota in Crohn's disease, liver cirrhosis and (morbid) obesity and explored its potential as a disease (progression) marker by applying various molecular, epidemiological and statistical approaches.

To study the fecal microbiota composition in patient groups, collection of high quality samples should be feasible for large (follow-up) cohorts. Therefore, the effect of different sampling and storage techniques on the fecal microbiota composition was evaluated in **Chapter 2**. As this may be affected by differences in fecal consistency (*i.e.* having diarrhea or constipation) samples were included from healthy subjects as well as from patients with either IBD or IBS. After determining optimal collection and sampling strategies, the intestinal microbiota was analyzed in three clinical entities that have previously linked to perturbations of the indigenous microbiota: Crohn's disease, obesity and liver cirrhosis.

The role of the intestinal microbiota in relation to disease activity in Crohn's disease was investigated in **Chapter 3**. As many studies are hindered by a cross-sectional design, we conducted a prospective follow-up study to investigate whether the fecal microbiota changes over time in relation to disease activity by 16S amplicon sequencing. Subsequently, we performed a proof of principle study to assess if we could use the fecal microbiota as a marker for disease activity in **Chapter 4**. As at present, no specific bacterial species or genera have been identified that enable the discrimination between active and remission patients, we applied machine learning techniques to identify a bacterial profile with an optimal discrimination between active and remission samples of patients with Crohn's disease. Next to the microbiota composition, volatiles in exhaled breath are shown to be indicative for various diseases and might therefore be useful as a biomarker in Crohn's disease. As the microbiota produces various volatile metabolites which can be excreted in feces, but also in exhaled breath, we investigated the relation between the intestinal microbiota and volatile metabolites in breath of Crohn's disease patients in **Chapter 5**.

In **Chapter 6,** we applied MiSeq sequencing to investigate the impact of sleeve gastrectomy on the fecal microbiota of obese individuals and examined whether changes in microbiota composition were associated with markers of inflammation, glycemic control and intestinal barrier function. Although this technique is the current state of the art to assess the microbi-

ota composition, next-generation sequencing is not yet applicable in the clinic. Therefore, we investigated the fecal and mucosa-associated microbiota of patients with compensated and decompensated liver cirrhosis using the rapid and easily implementable IS-profiling technology in **Chapter 7**. In this study, we focused on the impact of disease severity as indicated by the Child Pugh class to evaluate whether the microbiota may have potential as disease progression marker. Finally, a general discussion is presented in **Chapter 8** where we will summarize the most important results and discuss potential future research.

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The effect of sampling and storage on the fecal microbiota composition in healthy and diseased subjects

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ABSTRACT

Large-scale cohort studies are currently being designed to investigate the human microbiome in health and disease. Adequate sampling strategies are required to limit bias due to shifts in microbial communities during sampling and storage. Therefore, we examined the impact of different sampling and storage conditions on the stability of fecal microbial communities in healthy and diseased subjects. Fecal samples from 10 healthy controls, 10 irritable bowel syndrome and 8 inflammatory bowel disease patients were collected on site, aliquoted immediately after defecation and stored at -80°C, -20°C for 1 week, at +4°C or room temperature for 24 hours. Fecal transport swabs (FecalSwab, Copan) were collected and stored for 48-72 hours at room temperature. We used pyrosequencing of the 16S gene to investigate the stability of microbial communities. Alfa-diversity did not differ between all storage methods and -80°C, except for the fecal swabs. UPGMA clustering and principal coordinate analysis showed significant clustering by test subject ($p<0.001$) but not by storage method. Bray-Curtis dissimilarity and (un) weighted UniFrac showed a significant higher distance between fecal swabs and -80°C versus the other methods and -80°C samples ($p<0.009$). The relative abundance of *Ruminococcus* and Enterobacteriaceae did not differ between the storage methods versus -80°C, but was higher in fecal swabs ($p<0.05$). Storage up to 24 hours (at +4°C or room temperature) or freezing at -20°C, did not significantly alter the fecal microbial community structure compared to direct freezing of samples from healthy subjects and patients with gastrointestinal disorders.

INTRODUCTION

The gastrointestinal microbiota is the most complex and densely populated ecosystem colonizing the human body. This indigenous microbiota confers several important beneficial functions to its host, including the protection against invading pathogens, stimulation of gut maturation and immune development and homeostasis, and the metabolism of nutrients and xenobiotics. [1].

An altered microbiota composition (dysbiosis) has been associated with a wide range of diseases, such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), but also with metabolic diseases such as non-alcoholic fatty liver disease and type-2 diabetes [2–4]. Recent introduction of high-throughput sequencing methods has paved the way for the discovery of key microbial players in the pathophysiology of these diseases. Although differences in composition and metabolic activity have been clearly reported, the causal contribution of such key bacterial species or groups is in most cases not yet clear. This may be due to the relative small study populations, different disease phenotypes, and lack of control for potential confounding factors such as medication, co-morbidity and differences in dietary patterns. Therefore, large well-defined prospective cohorts are required to gain further insight in the role of the microbiota in heterogeneous disease entities. To accurately analyze the fecal microbiota, it is crucial that the sampling and storage procedures do not alter the microbial composition. Therefore, sampling methods that are applicable in large cohorts and do not bias the results are needed for these studies. Although immediate freezing of fecal samples is generally considered the gold standard, this is logistically challenging in large-scale studies. Moreover, thawing of frozen samples during transport is known to have a dramatic effect on DNA integrity [5]. A limited number of studies have examined the effect of different storage methods on the fecal microbiota.

Lauber *et al.* showed that the phylogenetic structure and diversity of bacterial communities in fecal samples were not influenced by storage at room temperature for 14 days compared to aliquots immediately stored at -20°C [6]. However, three other studies demonstrated a significant change in fecal microbiota composition after 24 hour storage at room temperature, but not when frozen within 24 hours [5,7,8]. Apart from storage at room temperature, cooled storage is also feasible in large cohorts. Wu *et al* investigated the storage of fecal samples on ice and showed that such storage for up to 48 hours did not result in significant differences in the fecal bacterial community [9].

The abovementioned studies showed only a small impact of different storage methods on microbiota composition, but the conclusions drawn were based on very small sample sizes including as little as 2 to 4 subjects.[5–9]. Moreover, the vast majority of these studies focused on fecal samples from healthy subjects. It is however conceivable that the effect of sample and storage collection methods differentially affects the microbiota composition in patients,

especially in those with altered bowel habits or in patients on medication. Data on the influence of sampling methods on the microbiota of patients with gastrointestinal disorders is lacking. Along with storage temperature, other user friendly sampling methods should be investigated, which are relatively easy to implement in large cohorts or when including consecutive patients from daily clinical practice. Fecal swabs are routinely used in clinical settings to detect enteropathogens and multidrug resistant Enterobacteriaceae, proving the feasibility of this method [10,11]. Since it is claimed that some of these swabs are also suitable for molecular analysis, it is conceivable that they might also be usable to study the fecal microbiota composition by means of next generation sequencing. However, to the best of our knowledge, no study has explored the fecal swab as a possible sampling method to characterize the fecal microbiota yet. The aim of the present study is to investigate the effect of different sampling and storage methods on the fecal microbiota composition in both healthy and diseased subjects.

MATERIAL AND METHODS

Study population

Ten healthy individuals (HC), 10 IBS outpatients and 8 hospitalized IBD patients (4 ulcerative colitis (UC), 4 crohn's disease (CD) patients) were included in this study. IBS was diagnosed by the Rome III criteria. The IBD diagnosis was based on clinical and endoscopic or radiological findings conform the European Crohn's and Colitis Organisation (ECCO) guidelines. Disease activity was scored by the validated Harvey Bradshaw index (HBI) for CD patients and the Simple Clinical Colitis Activity Index (SCCAI) for UC patients [12,13]. Exclusion criteria for all subjects were severe co-morbidity and having a stoma or a total colectomy. Healthy subjects were asked to complete the Rome III questionnaire to exclude undiagnosed IBS. All subjects completed a questionnaire about their age, BMI, smoking status, medical history, medication use and the Bristol stool form scale [14].

Ethical statement

The subjects included in the present study participated in the ongoing IBDSL cohort study (*i.e.* the IBD patients) or the IBS cohort study (*i.e.* IBS patients and healthy controls) and gave written informed consent prior to participation. Both study protocols have been approved by the Maastricht University Medical Center+ Committee of Ethics and are executed according to the revised Declaration of Helsinki (59th general assembly of the WMA, Seoul, South Korea, Oct. 2008). The studies have been registered in the US National Library of Medicine (http://www.clinicaltrials.gov, NCT02130349 and NCT00775060, respectively).

Sample collection

Each subject provided a fresh stool sample in a stool container on site. Within 10 minutes upon defecation, the fecal sample was aliquoted and aliquots were immediately stored under different conditions: at -80°C (reference method), at -20 °C for 1 week (1wk -20 °C), at 4°C for 24 hours (24h +4°C) or at room temperature for 24 hours (24h RT). In addition to the effect of different storage temperatures, a fecal swab (Copan Italia S.P.A., Brescia, Italy), a commercially available transport swab with a preservation medium, often used in clinical practice, (further referred to as FecalSwab) was turned into the stool. The FecalSwabs were stored thereafter at room temperature for 48-72 hours to simulate the scenario of auto-collection by patients and/or study subjects and subsequent transport to the laboratory by mail. Thereafter, all samples were frozen at -80°C until further processing.

DNA isolation

PSP lysis buffer (Stratec Molecular, Berlin, Germany) was added to a sterile vial containing 0.5 g of 0.1 mm zirconia/silica beads and 4 3.0-3.5 mm glass beads (BioSpec, Bartlesville, USA). Frozen stool aliquots were added to the vials. The samples were homogenized in a MagNA Lyser

instrument (Roche, Basel, Switzerland) in three cycles of 1 min. at a speed of 5500 rpm. Samples were kept on ice for one minute in between cycles. DNA isolation was continued using the PSP Spin Stool Kit (Stratec Molecular, Berlin, Germany) according to the manufacturers' instructions. DNA was finally eluted in 200 µl elution buffer. The DNA quantity and quality was measured by NanoDrop ND-1000 (Thermo Scientific, Wilmington, USA). Samples with a DNA concentration less than 20 ng or an $A_{260/280}$ less than 1.8 were subjected to ethanol precipitation to concentrate or further purified, respectively, to meet the quality standards. Ten FecalSwab samples with a total DNA content of less than 400 ng were excluded from this research.

454 pyrosequencing

A total of 147 samples with a minimal concentration of 20 ng/µl and a 260/280 ratio of at least 1.8 were send to BGI (Hong Kong) for 454 pyrosequencing. Amplicon libraries for pyrosequencing of the 16S rDNA V1-V3 regions were generated using 534F (ATTACCGCGGCTGCTGG) and 27R (AGAGTTTGATCCTGGCTCAG) primers. A key of 10 nucleotides unique for each sample was used. The 454 sequencing run was performed on a GS FLX pyrosequencing system using Titanium chemistry (Roche, Branford, USA).

Enumeration of Methanobrevibacter smithii by qPCR

For those subjects of whom all aliquots were analysed by 454 pyrosequencing, samples were also subjected to an 5'-nuclease based real-time PCR assay for the enumeration of the archeon *M. smithii* targeting the 16S rRNA gene as described previously [15]. Amplifications were conducted in a total volume of 25 µL, containing 1× Absolute qPCR Mix (ABgene, Hamburg, Germany), 200 nM of forward and reverse primers, 200 nM TaqMan probe, and 2 µL of tenfold diluted target DNA. The amplification (2 minutes at 50°C, 10 minutes at 95°C, and 42 cycles of 15 seconds at 95°C and 1 minute at 60°C) and detection were conducted with an Applied Biosystems Prism 7900 sequence detection system (Applied Biosystems). Samples with threshold cycle (Ct)-values <40 were considered positive. Log10 DNA copies for a given archaeal species per gram of wet weight faeces was calculated for each stool sample from the Ct-values using a quantification scale ranging from 10-10^7 target copies.

Data analysis

The V1-V3 16S rDNA bacterial sequences that were used in this paper have been submitted to the EMBL databases under accession No. PRJEB6765. To reduce the error rate, raw pyrosequencing reads were passed through quality filters using Mothur version 1.32.1 [16]. Only sequences with a perfect proximal primer fidelity, a minimum average score of 25 over a window size of 50 nucleotides, a read length between 200 and 590, a maximum of one ambiguous base call and a maximum homopolymer length of 6 were retained for further analysis. Subsequent data processing was done by Quantitative Insights Into Microbial Ecology (QIIME) version 1.7 [17]. After de-multiplexing, sequences were clustered by the UCLUST algorithm into operational

taxonomic units (OTUs) based on 97% similarity against the Greengenes reference set version May 2013 [18]. Default parameters for UCLUST were applied apart for the following parameters: maxrejects=100 and stepwords=16. To reduce the influence of pyrosequencing errors, the creation of de novo OTUs for sequences that did not cluster to reference sequences was disabled. The following metrics of species richness and diversity within communities (alpha-diversity) were determined: observed OTUs (observed richness), Chao1 index (estimated richness), Shannon diversity index and Phylogenetic Diversity (PD) whole tree. Beta-diversity or diversity shared across samples was determined by the unweighted and weighted UniFrac distance and Bray-Curtis dissimilarity (BC) at a rarefaction depth of 3445 seq/sample. [19].

Statistical analysis

In order to examine the potential loss in species richness/diversity during storage, pairwise comparisons were made for the alpha diversity metrics between the aliquots stored according to the reference method (direct storage at -80°C) and each of the other storage methods by means of the Wilcoxon signed rank test. To determine the effect of storage on the shared diversity between samples, beta diversity metrics (Bray-Curtis dissimilarity, unweighted UniFrac and weighted UniFrac) were calculated for the aliquots stored at -80°C versus each of the other storage methods (*i.e.* -80°C–1wk 20°C, -80°C–24h +4°C, -80°C–24h RT, and -80°C–FS). Subsequently these distances (within subjects across sampling methods) were tested by means of the Wilcoxon signed rank test. The Mann-Whitney U test was used to compare the distances of the reference methods between subjects. Statistical differences in community structure among test subjects and sampling and storage method were tested using PERMANOVA. The G-test was used to test associations between OTUs presence and storage method. To correct for multiple testing a false discovery rate (FDR) of 0.25 was used as a cut off value. To examine whether storage methods had a significant impact on the recovery of oxygen sensitive microorganisms, pairwise comparisons of the relative abundance of *Ruminococcus, Faecalibacterium, Roseburia* and *M. Smithii* was performed by the Wilcoxon signed rank test. In accordance, differences in the relative abundance of Enterobacteriaceae was also examined by the Wilcoxon signed rank test since the Cary-Blair medium of the FecalSwab is known to enhance the recovery of enteropathogenic bacteria [20]. Statistical analysis were performed using SPSS version 19 and QIIME 1.7.

RESULTS

Study population

A total of 28 study subjects, 10 healthy individuals, 10 IBS patients and 8 IBD patients (18-71 years) were included in this study. Half of the IBS patients were diagnosed with diarrhea pre-dominant IBS (n=5), followed by mixed IBS (n=4) and constipation pre-dominant IBS (n=1). None of the study subjects used probiotics, whereas one IBS patient followed a strict vegetarian as well as a gluten free diet at the time of the study. Among the IBD patients, 4 patients suffered from Crohn's disease and 4 patients had ulcerative colitis. All except one IBD patient were experiencing an exacerbation at the time of fecal sampling. Baseline characteristics of all study participants are presented in table 2.1.

454 sequencing

A complete set of samples (*i.e.* immediately stored at -80°C, stored at -20 for 1 week, stored at +4°C for 24 hours, stored at room temperature and a FecalSwab) was obtained from 28 individuals. Ten out of 28 FecalSwab samples were excluded for 16S V1-V3 library construction due to an insufficient DNA yield. In total, 1,825,747 raw sequences were obtained from sequencing all remaining samples. After quality filtering and binning, a total of 927,825 sequences with an average of 7,137 sequences per sample (range 1,066-25,887 sequences/sample) remained for further analysis. A total of 6,105 OTUs were detected with a mean Good's coverage of 92 ± 0.03%. The microbiota of the fecal samples stored according to the reference method was dominated by Bacteroidetes (mean 50.4%, SD 0.17%) and Firmicutes (mean 42.1%, SD 0.17%), followed by Proteobacteria (mean 4.6%, SD 5.3%). No significant differences in the relative abundance of the dominant phyla were found between the reference method and other sampling and storage methods. The microbial profile at the genus level within subjects was rather similar for aliquots stored under the different conditions (figure 2.1).

Effect of storage methods on diversity and richness

The Chao1 richness estimates and Shannon diversity indices did not differ statistically significantly between samples stored directly at -80°C and the aliquots from the same samples stored at -20°C for one week ($p=0.829$ and $p=0.456$, respectively), 4°C for 24 hours ($p=0.139$ and $p=0.838$, respectively) and room temperature for 24 hours ($p=0.946$ and $p=0.466$ respectively). The FecalSwab however, showed a statistically significantly higher Chao1 estimate and Shannon index ($p<0.01$ and $p<0.01$, respectively) than the reference method. The Chao1 richness and Shannon diversity indices for subjects of whom all aliquots were available for analysis (n=17) are depicted in figure 2.2. Of note, the -20°C sample of HC2 showed higher Chao1 and Shannon estimates as compared to the other aliquots of this subject. No such differences were observed for the other subjects. Pairwise comparisons of other alpha diversity indices (observed richness and PD-whole tree) also showed a significantly higher diversities in the FecalSwab as compared

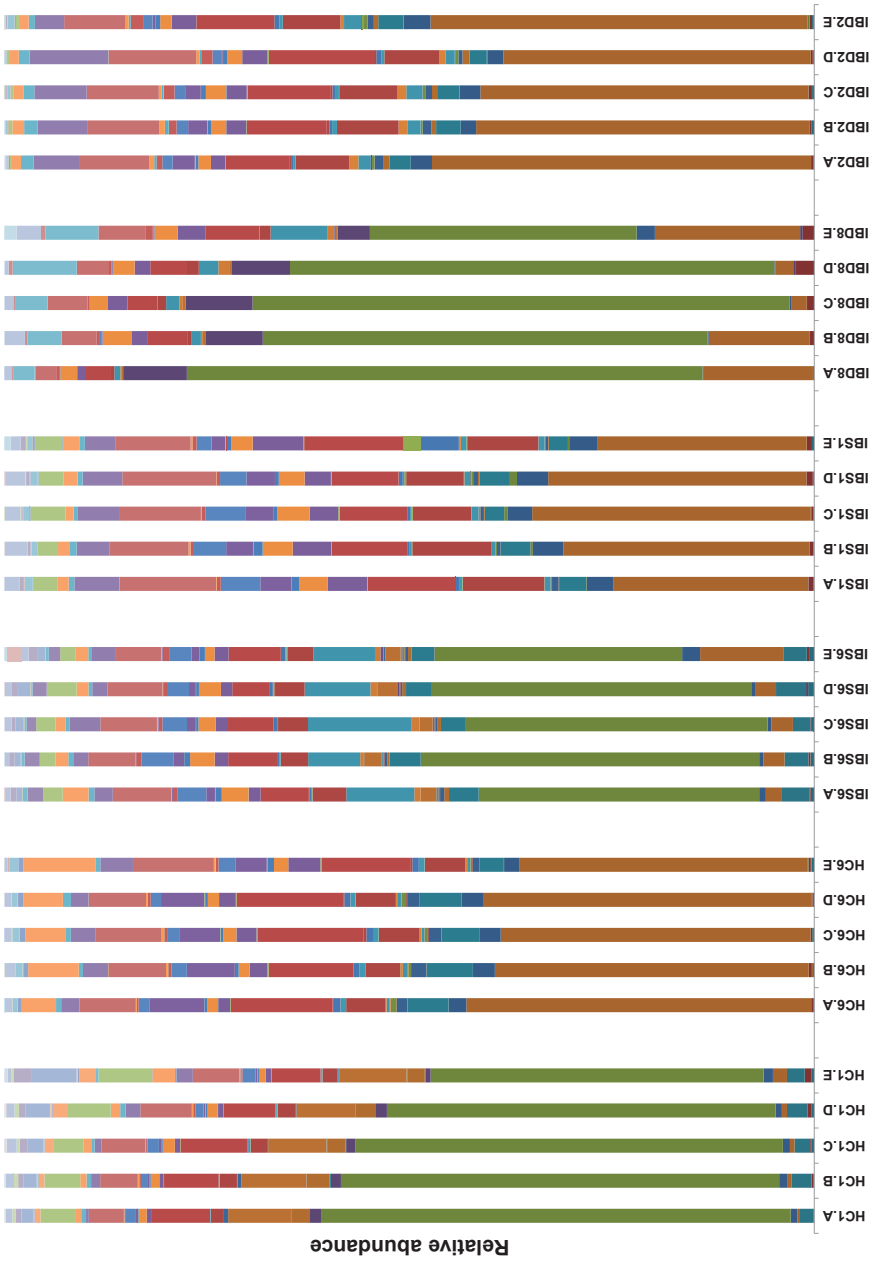
to the aliquots stored at -80°C, but no significant differences were found between this reference method and other storage methods (table 2.2).

Table 2.1. Characteristics of study population.
Continuous variables are presented as median and range.

	HC (N=10)	IBS (N=10)	IBD (N=8)
Age (years)	25.5 (23-44)	51.5 (23-71)	55.5 (20-67)
Male (%)	60	60	37.5
BMI	21.3 (19.2-24.8)	23.8 (18.1-34.7)	27.6 (35.3-22.7)
Smoker (%)			
Currently	0	20	0
Stopped	10	10	50
Never	90	70	50
Stool consistency (Bristol Stool)			
Type 1-2	0	3	0
Type 3-4	10	3	0
Type 5-7	0	4	8
Disease phenotype[1]	n.a.	IBS-D: 5 IBS-C: 1 IBS-M: 4	4 CD 4 UC
Disease location	n.a.	n.a.	colonic: 1 left sided:3 ileocolonic: 2 pancolitis:1
Medication (n)[2]	3 Birth control: 2 Ironsupplement and asthma medication:1	8 Birth control: 2 Motility: 3 Antidepressant: 4 PPI: 1 Analgesics: 3	9 TNF-α: 3 Thiopurine: 2 Aminosalicylate: 2 Methotrexate: 1 Glucocorticoid: 6 Antibiotics: 2
Abdominal surgery	0	4[3]	2[4]
Active disease[5]	n.a.	n.a.	CD: 4/4 UC: 3/4

[1] CD: Crohn's disease, UC: ulcerative colitis, IBS-D: diarrhea predominant IBS, IBS-C: constipation predominant IBS, IBS-M: mixed IBS
[2] Patients took multiple medications.
[3] 2 subjects had a hysterectomy, 1 subject had a sigmoid resection, 1 subject underwent an appendectomy
[4] 1 subject had a hysterectomy, 1 subject had a hysterectomy, appendectomy and illeocecal resection
[5] Active disease if Harvey-Bradshaw Index (HBI)>4 (CD) or Simple Clinical Colitis Activity Index (SCCAI)>3 (UC)

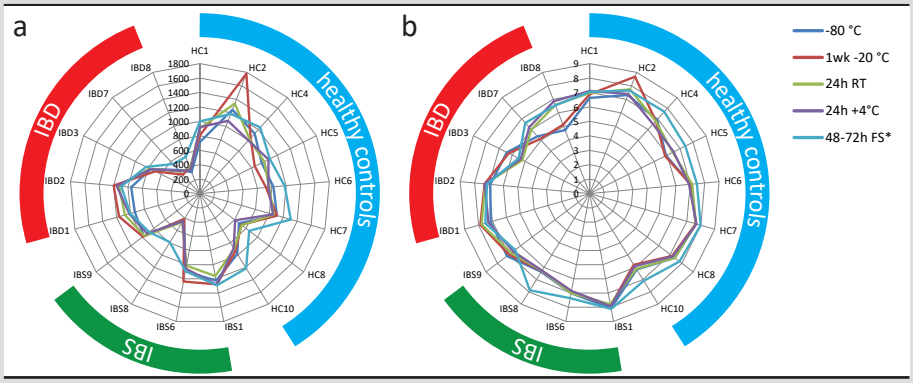
Figure 2.1. Relative distribution of bacterial taxa at genus level per storage or sampling method for a representative set of test subjects (2 healthy controls, 2 IBS and 2 IBD patients).



Letters indicate the storage method (A=-80°C, B=1wk -20°C, C=24h +4°C, D=24h RT, E=FecalSwab)

Figure 2.1. Continued.

Figure 2.2. Effect of storage methods on the alpha diversity.



Chao 1 richness estimate (a) and Shannon index (b) is shown at the y axis and test subjects are shown at the x axis. The blue, green and red bands indicate healthy test subjects, IBS and IBD patients respectively. Only test subjects (17/28) with a complete set of samples (5 different sampling and storage methods) available for analysis are shown. *p<0.01 compared with -80°C as determined by the wilcoxon signed rank test.

Effect of storage on the fecal microbial structure

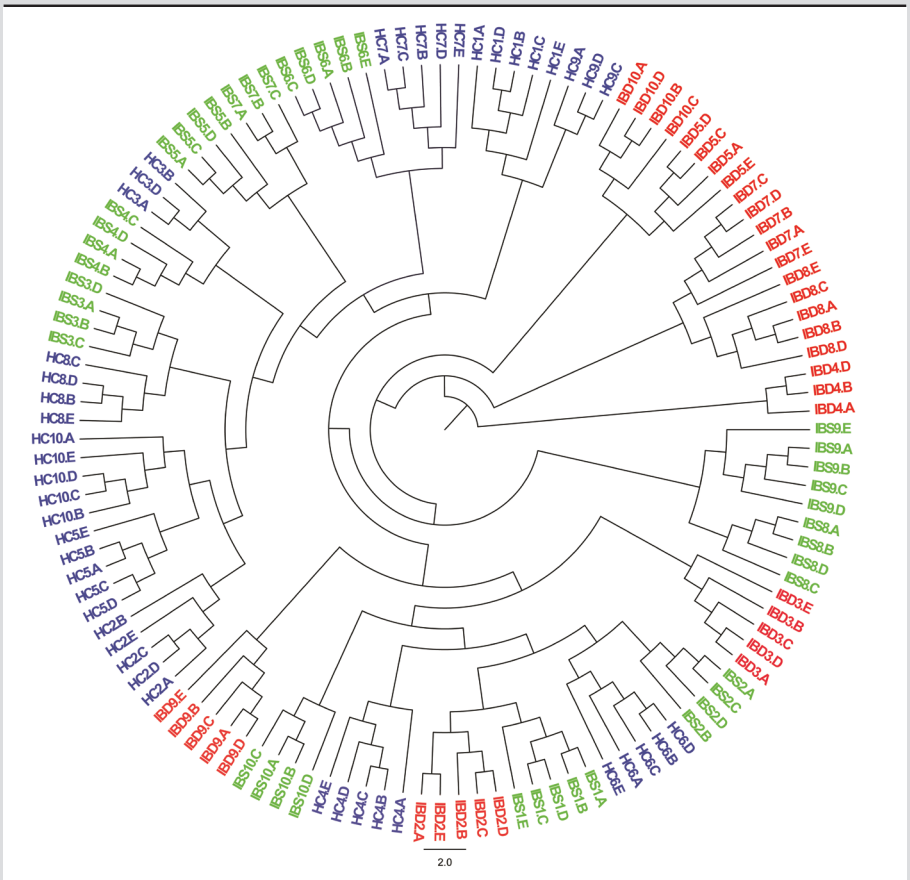
UPGMA clustering based on unweighted pair-wise UniFrac distances between all samples resulted in separation of the samples based on test subjects as shown in figure 2.3. In line with the above findings, principal coordinate analysis of unweighted UniFrac distance showed a strong clustering of the samples based on test subjects, although the FecalSwabs seems to deviate most from its original subject specific cluster (supplemental figure S2.1, S2.2 and S2.3). Principal coordinate analysis of the weighted UniFrac distances and Bray-Curtis dissimilarity showed similar results (supplemental figure S2.4-S2.9). Moreover, applying PERMANOVA revealed that samples between subjects were statistically significantly different from each other ($p<0.001$ for unweighted UniFrac, weighted UniFrac and Bray-Curtis). No significant clustering was found based on the different sampling and storage methods by PERMANOVA.

To examine the impact of individual storage methods in more detail, we subsequently examined the beta-diversity distances between the reference storage method and each of the other storage methods. The median and interquartile unweighted UniFrac distances are depicted in figure 2.4.

The between-subject unweighted UniFrac distance of all samples stored at -80°C (reference method) was statistically significantly larger than the within-subject unweighted UniFrac distances between the reference method and each of the other storage methods ($p<0.05$). However, paired comparisons demonstrated a statistically significantly larger unweighted UniFrac distance between the reference method and the FecalSwab as compared to the distance between the reference method and all other storage methods as seen in figure 2.4.

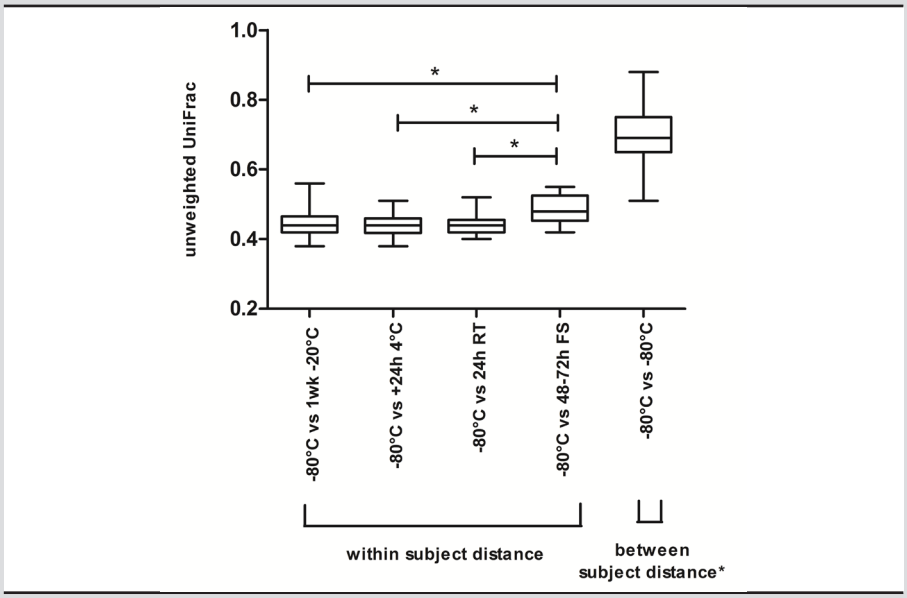
Pairwise comparisons of the weighted UniFrac distance and Bray-Curtis dissimilarity also showed that the beta diversity distances between the FecalSwab and the reference method is statistically significantly larger compared to the reference method and all other storage methods (supplemental figure S2.10 and S2.11). The median unweighted UniFrac distances of -20°C, +4°C and RT versus the reference method were comparable between each other (figure 2.4), implying that aliquots stored under these conditions were equally similar to samples stored directly at -80C. This is also shown when pairwise comparisons were made of the weighted UniFrac and Bray-Curtis dissimilarity (supplemental figure S2.10 and S2.11). The effect of the different storage methods was not significant in the subgroups of healthy subjects, IBS and IBD patients.

Figure 2.3. UPGMA tree based on unweighted UniFrac distance.



All subjects (n=28) are included. Letters indicate the storage method (A=-80°C, B=1wk -20°C, C=24h +4°C, D=24h RT, E=FecalSwab). Samples of healthy subjects (HC), IBS and IBD patients are written in blue, green and red, respectively.

Figure 2.4. Box-and-whisker plot of unweighted UniFrac distance between reference method and other sampling and storage methods.



Whiskers express the maximum and minimum values. *indicate a significant difference (p<0.01).

Table 2.2. Effect of sampling and storage methods on alpha diversity metrics. Median and range are shown in the table. (*p<0.05 compared to -80°C)

Diversity indices	Sampling & storage methods				
	-80 °C	**1w -20 °C**	**24h RT**	**24h +4°C**	**48-72h FecalSwab**
	(n=28)	(n=27)	(n=28)	(n=28)	(n=18)
Observed species	540.1 (112.5-811.2)	545.5 (93.8-995.9)	549.2 (95.3-830.6)	529.1 (90.6-791.4)	593.9* (280.8-746.9)
Chao1	955.8 (206.0-1457.3)	935.2 (143.6-1780.6)	963.8 (180.1-1525.6)	936.36 (130.1-1440.3)	1042,62* (544.4-1308.4)
Shannon	7.0 (4.4-8.4)	6.9 (4.1-8.7)	7.0 (4.1-8.4)	6.9 (4.4-8.5)	7.4* (5.5-8.1)
PD whole tree	27.5 (8.6-37.3)	27.5 (6.8-46.0)	28.4 (7.8-37.7)	27.6 (7.0-37.2)	30.0* (14.5-37.3)

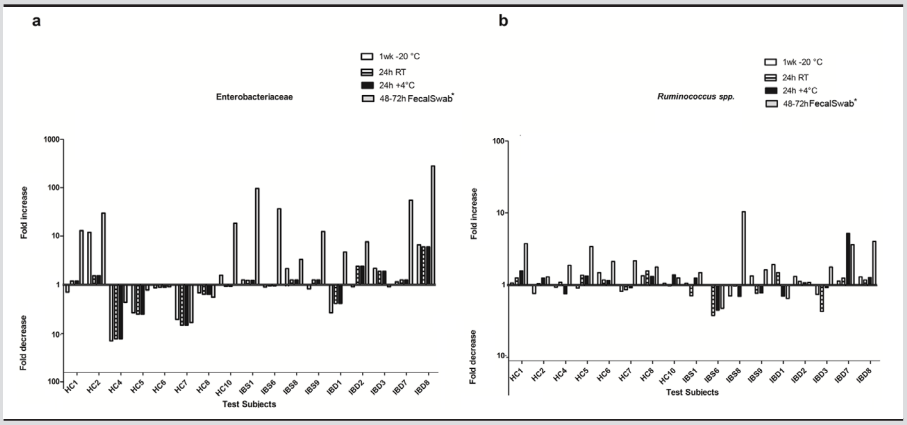
Effect of sampling and storage on the presence and relative abundance of specific bacterial taxa

No association between the presence of specific bacterial taxa and the different storage methods was found as examined by applying the G- test with a cut-off value of q=0.25. Subsequently, the relative abundances of *Faecalibacterium*, *Roseburia* and *Ruminococcus* were examined since these species are known to be extremely sensitive to oxygen and thus different sampling and

storage methods. No significant differences were found between samples stored at -80°C and the other sampling and storage methods in the relative abundance of *Faecalibacterium* and *Roseburia.* Remarkably, the relative abundance of *Ruminococcus* showed a significant increase in the FecalSwab compared to the -80 °C samples ($p<0.05$), but not in the other storage methods (figure 2.5). In addition to the extreme oxygen-sensitive species, the relative abundance of Enterobacteriaceae was examined. Paired comparisons showed a significantly higher abundance of Enterobacteriaceae in FecalSwab ($p<0.05$), but not in the other storage methods, when compared to the reference method.

Quantification of the archeon *M. smithii* revealed that in case of a positive result for the aliquot stored at -80°C (n = 9/18), all other aliquots (20°C, +4°C and RT) gave a positive PCR as well. Morover, the concentration of *M. smithii* in positive reference samples (median = 8.54; range 6.70-10.63 log10 DNA copies/g faeces) did not differ from the concentration in the other aliquots. However, the FecalSwab samples were PCR negative in the majority of subjects of whom the reference sample was positive (n=7/9) due to the strong diluting effect of the preservation buffer.

Figure 2.5. Fold change of relative abundance of Enterobacteriaceae (a) and *Ruminococcus spp.* (b) compared to -80°C.



Only test subjects (17/28) with a complete set of samples (5 different sampling and storage methods) available are shown. Fold changes are shown at the y axis (logarithmic scale). *Relative abundance in FecalSwabs was significantly higher as compared to reference storage method ($p<0.05$ as determined by Wilcoxon signed rank test).

DISCUSSION

The aim of this study was to investigate the effect of different sampling and storage methods on the stability of the fecal microbiota composition in healthy and diseased subjects. Previous studies have investigated the effect of various storage temperatures on the fecal microbiota composition and showed overall no significant differences in richness, diversity and clustering. These studies were however based upon a limited number of subjects, included only healthy individuals or did not report the health status of included subjects. As such, the generalizability of their results and conclusions is unclear [5–9]. Our study included both healthy as well as diseased subjects; diagnosed with common GI disorders that are reported to be associated with the intestinal microbiota, have differences in fecal consistency and use a variety of medications which enhances the heterogeneity of our study population and thereby the external validity of our results. No significant differences were found in the amount of observed OTUs, richness and diversity between the reference storage method and other methods. Similar results were observed in the total study population, as well as in the subgroups of healthy subjects, IBS and IBD patients.

Our results are in line with most previous studies, which showed no significant change in the overall microbiota composition between frozen fecal samples and samples stored at room temperature or 4 °C for 24 hours [5–9]. Only one previous study compared the microbial richness of directly frozen samples and samples stored at room temperature up to 24 hours and showed no statistically significant differences [8], which is in accordance with our findings. Previous studies were mainly performed in small numbers of healthy subjects or with unknown health status. We showed that the findings are also applicable to patients with different disease states, medication use and/or fecal consistencies. In contrast to the study of Roesch et al, which included four healthy subjects, we did not observed a decrease in the relative abundance of the extreme oxygen sensitive species *Faecalibacterium* and *Ruminococcus* or an increase in the relative abundance of Enterobacteriaceae in the fecal samples stored at room temperature for 24 hours as compared to the reference method in our cohort of 28 study objects. In addition to these results, we found that the relative abundance of extreme oxygen sensitive species *Roseburia* and the Firmicutes/Bacteroides ratio was not significantly different between the storage methods and reference method. Our results indicate only a minimal effect of the investigated storage methods on the fecal microbiota composition from the total population and all subgroups investigated. This observation is further supported by the UPGMA clustering and principal coordinate analyses, which showed that the fecal aliquots clustered based on test subject and not storage methods. Although the distances in beta diversity are relatively small and comparable between each and every storage method, we still recommend using a single standardized method within one study to minimize any potential sampling bias.

The variations seen within individuals are at least partly explained by the sequencing depth. We are aware that our sequencing depth is not ultradeep, therefore low abundant OTUs will not always be detected. However, the conclusions of our study did not change when we performed a subanalysis with the samples that had a higher sequencing depth (rarefaction at 5,000 seq/sample) (supplemental figure S2.12-S2.14 and supplemental table 2.1.).

In the present study FecalSwab samples were included to examine whether this sampling strategy, often used in the clinical setting, is also suitable for microbiota analysis. The FecalSwabs were stored for 48-72 hours at room temperature in modified Cary-Blair medium, which promotes survival of obligate anaerobes by the low oxidation reducing potential and is used for the isolation of enteric pathogens [20]. FecalSwabs showed a significantly higher microbial richness and diversity. This might be explained by a slight growth advantage of certain low abundant bacterial taxa, particularly within the significantly enriched Enterobacteriaceae and Ruminococcaceae families, in this medium. Such low abundant taxa, undetected at the applied sequencing depth in the other aliquots, could then rise above the detection limit. Although FecalSwabs still strongly clustered to the other aliquots of the same subject, this could also explain why the microbial community structure of the FecalSwabs showed a larger distance (Bray-Curtis dissimilarity and UniFrac distances) to the reference method than the other storage methods. The use of fecal swabs seems to be the most user friendly method to obtain fecal samples, however, in this study 10 out of 28 FecalSwab samples yielded insufficient DNA quantity and could therefore not be sequenced. When using FecalSwabs, an adapted DNA isolation protocol (*e.g.* not depending on column-based purification) is recommended to retain highest possible DNA yield. The results of the FecalSwab are only applicable to this particular swab and other transport swabs containing Cary-Blair medium and should not be extrapolated to swabs using different transport medium. It is important to note that within or between studies, the results of the FecalSwab should not be compared with other feces collection and storage methods due to the variations in the microbiological structure between these sampling methods. To our best knowledge this is the first study that investigates the effect of different sampling and storage methods by means of pyrosequencing within a rather large, heterogeneous group. By including healthy subjects and those with a GI disorder, the results drawn from this study can be generalized to other populations. The results and conclusions are only applicable for studies on the taxonomic composition of the fecal microbiota. Metagenomics, metatranscriptomics, metaproteomics, and metametabolomics are becoming increasingly important in the study and understanding of the human GI microbiota. Metabolites, proteins and especially mRNA are known to be susceptible to enzymatic degradation. Therefore, studies on the impact of different sampling and storage methods on the stability of the metagenome metametabolome, metatranscriptome and metaproteome are also warranted.

Based on our and previous observations, we conclude that fecal samples stored at -20°C for 1 week, at +4°C for 24 hours and at room temperature for 24 hours retained highly similar bacterial structure at DNA level as fecal samples stored in the -80°C within 10 minutes. Direct freezing of the feces (*e.g.* in the home freezer of participating subjects) increases the potential for additional analyses (*e.g.* metatranscriptomics, metaproteomics), but thawing of the samples during transport should be prevented at any cost [8]. If direct freezing is not feasible within the setting of a study, we recommend storing feces at either room temperature or at +4°C (in a refrigerator) for a maximum of 24 hours.

When using the FecalSwab as a sampling method, the isolation procedure should be modified to ensure high DNA quality and quantity. Moreover, transport time (storage at room temperature) should be limited as the microbial community structures in the FecalSwabs (after 48-72h at room temperature) deviated most from the original microbial community structure. Homogenization of the feces prior processing appears not necessary since recent work showed that homogenization does not alter the fecal microbiota [21]. We recommend applying a single sampling and storage method within a study to prevent potential bias in the results.

ACKNOWLEDGEMENTS

The authors would like to thank Tim van den Heuvel and Zlatan Mujagic for their assistance.

CONFLICTS OF INTEREST

The authors declare no conflict of interest. A. Masclee receives grants from DSM, Grunenthal, Abbott and Danone. M. Pierik acted as a consultant for Takeda in the past, is a former lecturer for Abbvie, Falk, MSD and Ferring. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

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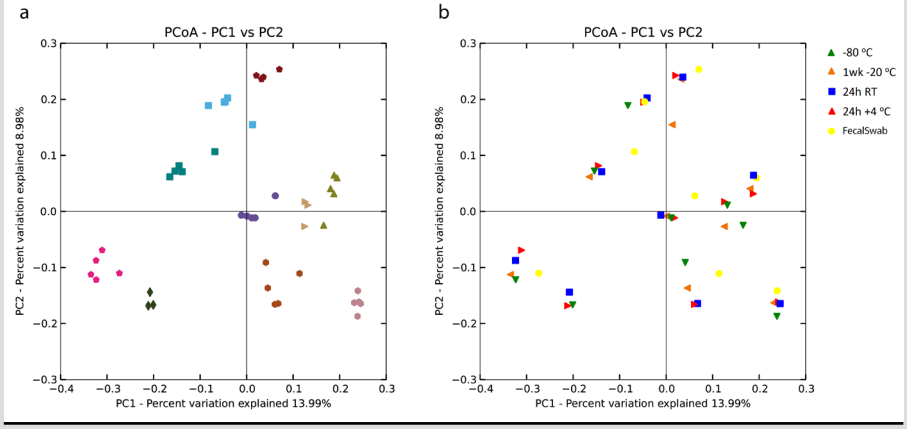
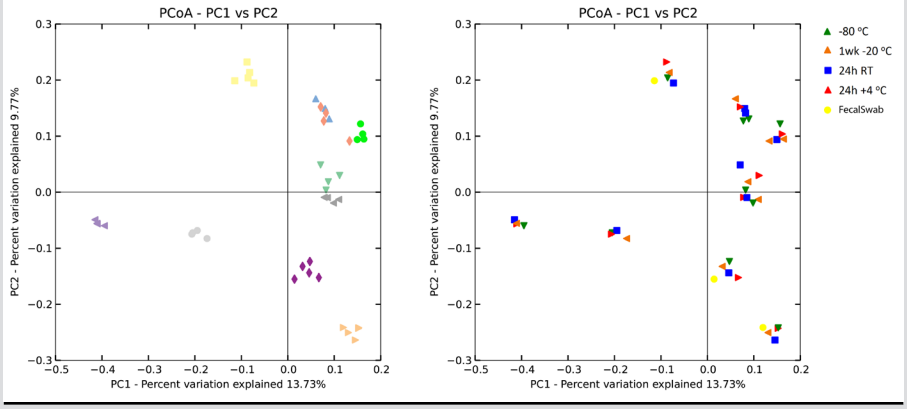
SUPPLEMENTAL INFORMATION

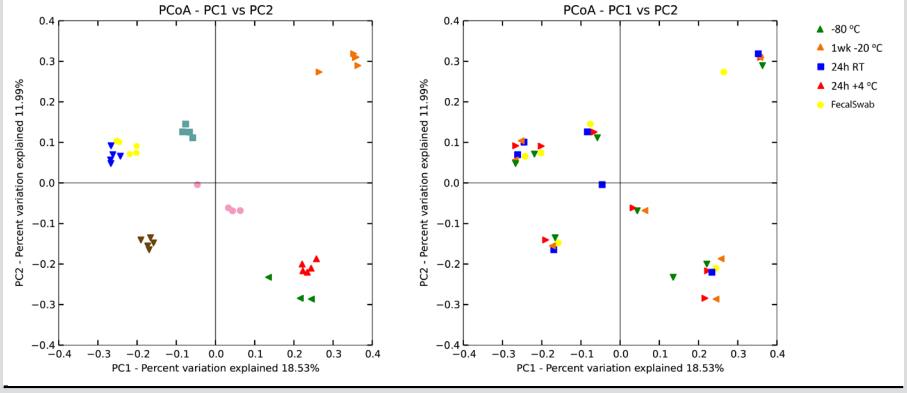
S2.1. Principal coordinate analysis based on unweighted UniFrac distance for healthy subjects colored based on subject (a) and storage method (b).



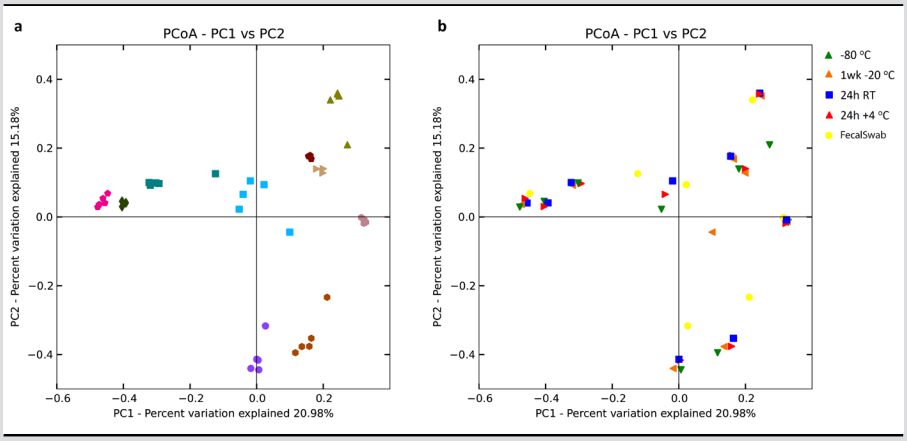
S2.2. Principal coordinate analysis based on unweighted UniFrac distance for IBS patients colored based on subject (a) and storage method (b).



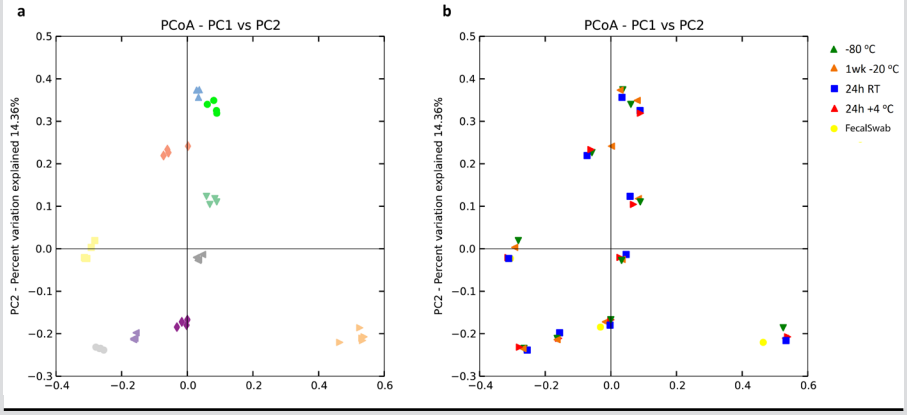
S2.3. Principal coordinate analysis based on unweighted UniFrac distance for IBD patients colored based on subject (a) and storage method (b).



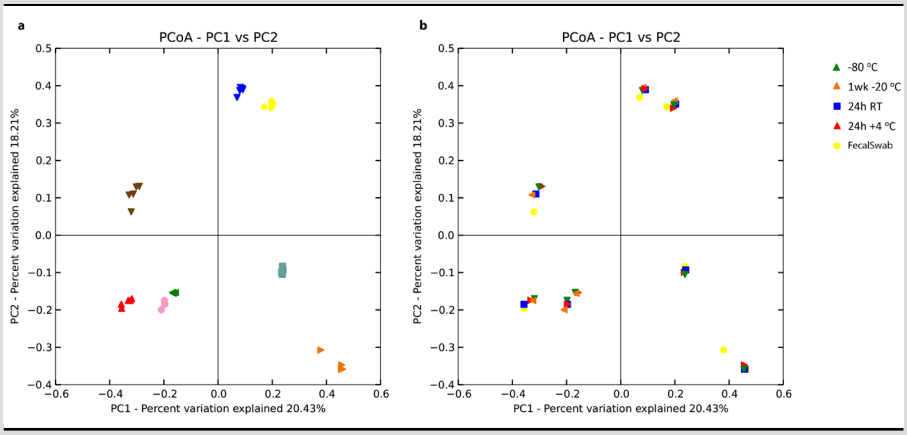
S2.4. Principal coordinate analysis based on Bray-Curtis distance for healthy subjects colored based on subject (a) and storage method (b).



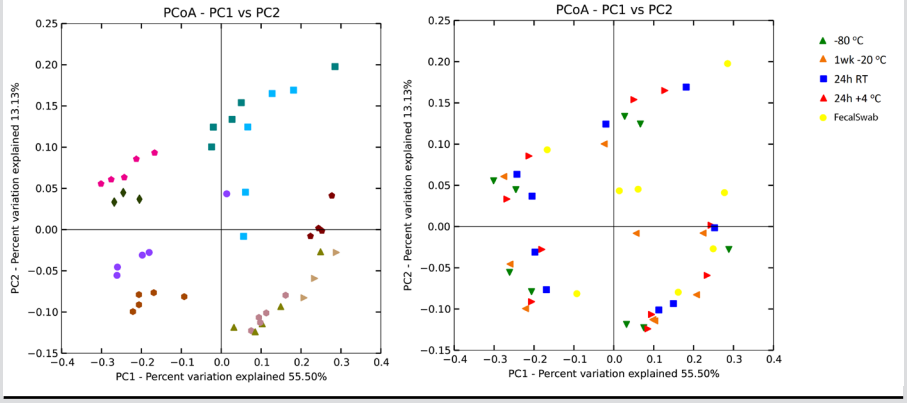
S2.5. Principal coordinate analysis based on Bray-Curtis distance for IBS patients colored based on subject (a) and storage method (b).



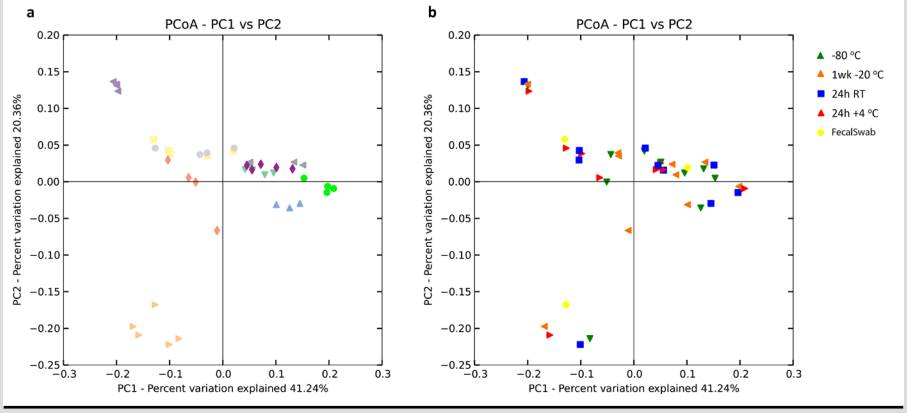
S2.6. Principal coordinate analysis based on Bray-Curtis distance for IBD patients colored based on subject (a) and storage method (b).



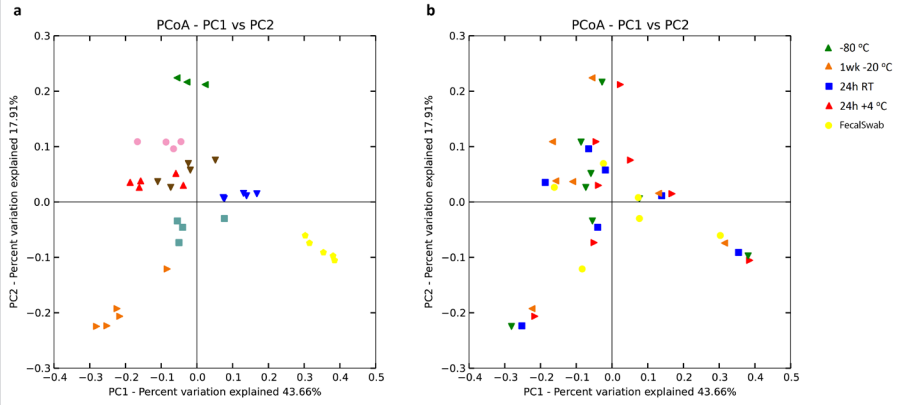
S2.7. Principal coordinate analysis based on weighted UniFrac distance for healthy subjects colored based on subject (a) and storage method (b).



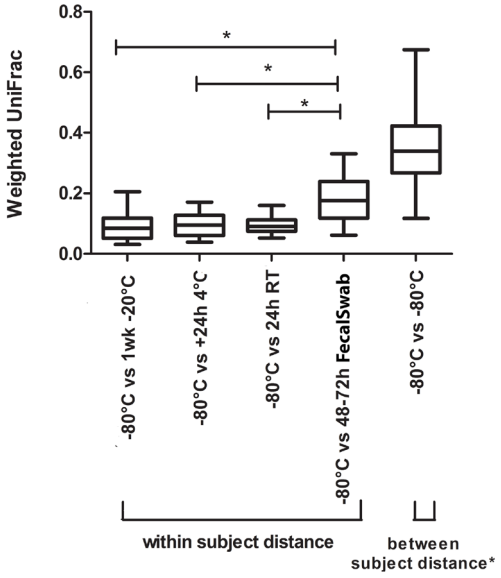
S2.8. Principal coordinate analysis based on weighted UniFrac distance for IBS patients colored based on subject (a) and storage method (b).



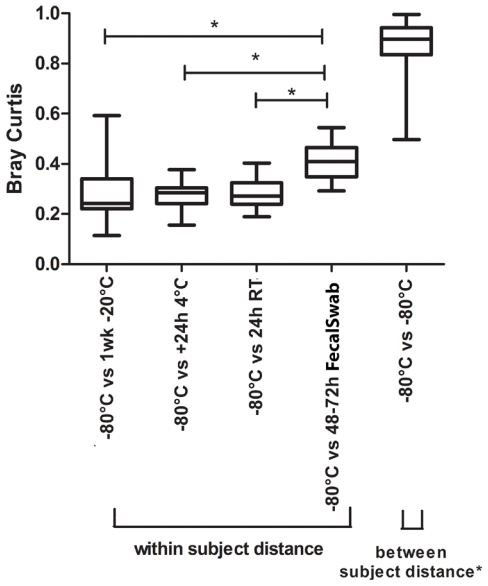
S2.9. Principal coordinate analysis based on weighted UniFrac distance for IBD patients colored based on subject (a) and storage method (b).



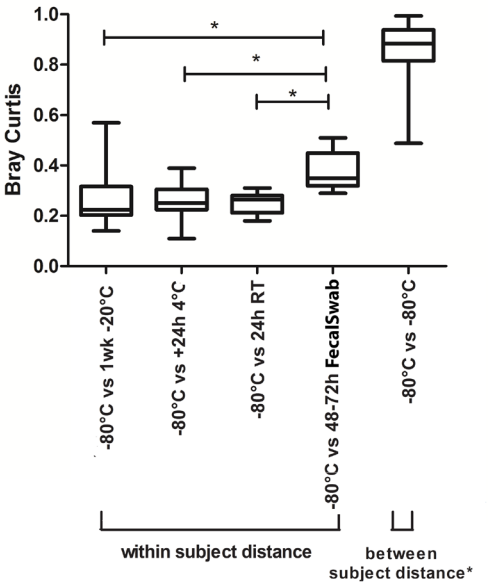
S2.10. Box and whisker plot of weighted UniFrac distance between reference method and other sampling and storage methods. (*p<0.05)



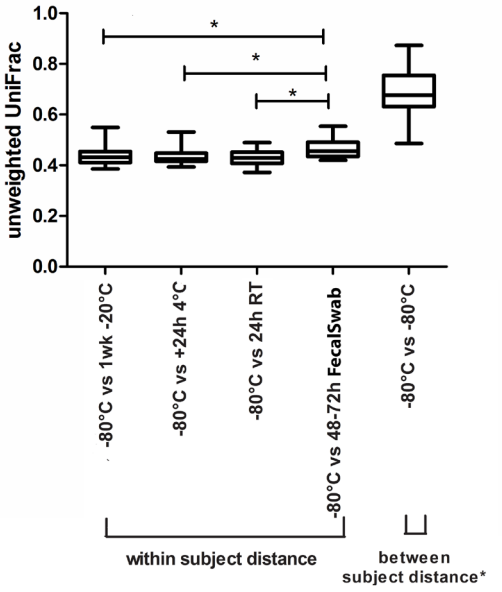
S2.11. Box and whisker plot of Bray-Curtis dissimilarity between reference method and other sampling and storage methods. (*p<0.05)



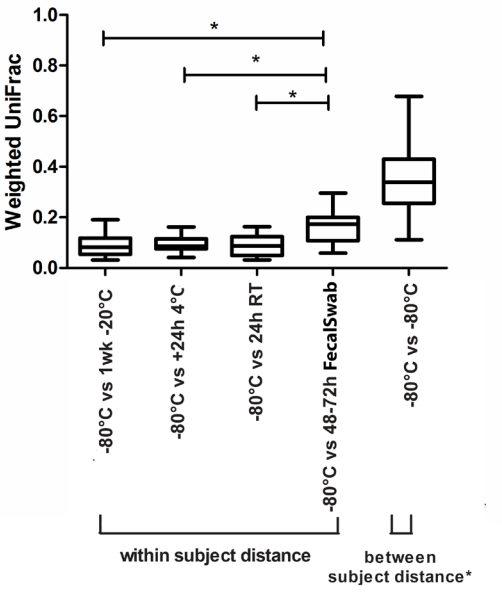
S2.12. Box and whisker plot of Bray-Curtis dissimilarity between reference method and other sampling and storage methods at a sampling depth of 5,000 seq/sample. (*p<0.05)



S2.13. Box and whisker plot of unweighted UniFrac distance between reference method and other sampling and storage methods at a sampling depth of 5,000 seq/sample. (*p<0.05)



S2.14. Box and whisker plot of weighted UniFrac distance between reference method and other sampling and storage methods at a sampling depth of 5,000 seq/sample. (*p<0.05)



Supplemental table S2.1. Effect of sampling and storage methods on alpha diversity metrics at a sampling depth of 5000 sequences/sample. Median and range are shown in the table. (*p<0.05 compared to -80°C)

Diversity indices	Sampling & storage methods				
	-80 °C (n=25)	1w -20 °C (n=21)	24h RT (n=22)	24h +4°C (n=22)	48-72h FecalSwab (n=13)
Observed species	699.0 (206.9-976.1)	691.8 (176.1-1212.4)	680.0 (244.6-1001.2)	662.2 (240.9-929.5)	760.1* (333.4-906.6)
Chao1	1176.0 (349.7-1683.5)	1202.3 (239.4-2039.6)	1135.8 (408.4-1816.9)	1182.6 (394.9-1575.6)	1271.2* (651.7-1522.5)
Shannon	7.0 (4.5-8.5)	7.0 (4.1-8.8)	7.1 (5.1-8.5)	6.9 (4.7-8.2)	7.2* (5.4-8.2)
PD whole tree	32.5 (10.5-42.0)	32.6 (9.1-52.9)	32.4 (11.7-43.0)	32.1 (12.0-40.6)	34.9* (15.4-42.4)

3

The stability of the fecal microbiota in Crohn's disease patients with changing disease course

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ABSTRACT

Background: Microbial shifts have been associated with disease activity in Crohn's disease (CD), but findings on specific taxa are not always consistent. This may be due to differences in populations included, molecular methods applied, potential confounders and the use of cross-sectional study designs. We aimed to prospectively examine the fecal microbiota, by means of next-generation sequencing, in adult well-characterized CD patients with either changing or stable disease course over time.

Methods: Fecal samples were collected at two time points from 15 healthy individuals (HC), 35 CD patients with stable disease course (remission) and 22 CD patients during remission and subsequent exacerbation. The microbial composition was assessed by sequencing of the V4-region of the 16S rRNA gene. The microbial diversity and richness of fecal samples was assessed by the Shannon index and PD whole tree and the observed species and Chao1 index, respectively. Stability of the microbiota composition within individuals as well as differences in the microbiota composition between individuals were assessed by Bray-Curtis dissimilarity and (un)weighted Unifrac distance.

Results: CD patients at baseline had a significantly lower microbial (median (IQR)) richness (Chao1 index (644.7 (440.1-817.2) and 800.4 (711.0-849.7), respectively; p=0.004) and diversity (Shannon index; 6.0 (5.0-6.4) and 6.3 (6.1-7.1), respectively; p=0.014) as compared to HC. However, the microbial richness and diversity did not significantly change over time in CD patients that subsequently developed an exacerbation when compared to HC and CD patients that remained in remission.

When assessing the overall microbial community structure at baseline, a subset of CD patients clustered apart from HC and was characterized by a low microbial diversity and a low relative abundance of *Faecalibacterium spp.* Moreover, the microbiota of both CD patients that maintained in remission (RR) and those that developed an exacerbation (RA) was less stable over time when compared to HC [(unweighted Unifrac median(IQR) RR 0.40(0.36-0.44), RA 0.38(0.36-0.42), HC 0.35(0.31-0.37)]. However, no differences in microbiota stability were observed between these two patient groups (p=0.39), nor was the stability impacted by medication use, surgery or disease localization.

Conclusion: CD patients showed a lower temporal stability than healthy controls, but this was not affected by differences in disease course. Furthermore, a subgroup of CD patients harbored a microbiota composition that deviated from the microbiota of HC, which warrants further investigation.

INTRODUCTION

Crohn's disease (CD), one of the two major subtypes of inflammatory bowel diseases (IBD), is a chronic gastrointestinal disease of which the incidence is increasing worldwide [1]. It is a relapsing disease characterized by periods of active inflammation with symptoms as abdominal pain and (bloody) diarrhea, alternated by periods of remission. The disease course varies between patients and has a poor predictability [2]. Treatment is mainly symptom-based and can be associated with mild to severe side effects [3]. In addition to obtaining relief of symptoms, current treatment strategies focus on mucosal healing and prevention of long-term complications [4]. CD has a significant impact on the patient's quality of life and exerts a heavy burden on the society due to medical costs and loss of work productivity, especially during periods of active inflammation [5,6].

Although the exact cause of CD is unclear, the generally accepted hypothesis is that CD results from an aberrant immune response against the commensal intestinal microbiota in genetically susceptible hosts. Previous studies reported perturbations in microbiota composition in CD patients, characterized by a decreased microbial diversity and changes in the relative abundance of specific taxa (*e.g.* reduction of *Faecalibacterium prausnitzii*, increase of Enterobacteriaceae) when compared to the microbiota of healthy individuals [7–11]. Moreover, several studies have also reported microbial shifts in relation to disease activity. For example, when compared to CD patients in remission, the microbiota of CD patients during an exacerbation is characterized by an increase of members of Enterobacteriacaea [12,13] and *Bacteroides* [14,15], and a reduction of *F. prausnitzii* [16–18] and *Clostridium coccoides* group [17,19] as indicated by several, but not all studies [19–22]. Inconsistencies between studies may in part be due to differences in the assessment of disease activity, applied molecular microbiological methods, and study populations but also to potential confounding factors such as medication use. Furthermore, the majority of these studies are based on a cross-sectional design comparing patients in remission versus those with active disease. Considering the inter-individual variation in microbiota composition and the heterogeneous nature of CD, longitudinal studies are particularly relevant. At least seven previous studies investigated the microbiota in adult CD patients in relation to changes in disease course over time [19,23–28]. Four of these studies focused on the predictive value of the microbiota on treatment response [23,24] or post-surgery recurrence [27,28], respectively. Three studies examined the microbiota in association to natural disease course [19,25,29], *i.e.* focusing on remission patients subsequently developing an exacerbation. All of these latter studies were of limited sample size and only one assessed the microbiota with next-generation sequencing techniques [25]. Although other techniques used in previous studies result in valuable information, they do not provide the same resolution as next-generation sequencing. To address these shortcomings, the present study was conducted. The aim of this study was i) to compare the fecal microbiota stability of 57 adult CD

patients with the fecal microbiota stability of healthy individuals and ii) to compare the stability of the fecal microbiota of CD patients with either changing or stable disease course over time by means of next-generation sequencing.

MATERIAL AND METHODS

Study population

A total of 57 CD patients and 15 healthy subjects were included in this study. CD was diagnosed based on clinical and endoscopic or radiological findings conform the ECCO guidelines [30]. CD patients of a prospective follow-up study [31] as part of the deeply phenotyped population-based IBDSL cohort [32], were available for analyses in the current study. For all patients, clinical data, blood and feces were collected at each visit to the outpatient clinic and during an exacerbation during one-year follow-up. As the current standard endoscopy is too invasive for disease monitoring over time and clinical indices do not correlate well with mucosal inflammation, disease activity was defined by the combination of fecal calprotectin (FC), serum C-reactive protein (CRP) and the Harvey Bradshaw Index (HBI). An exacerbation was defined by a FC concentration >250 µg/g or a FC concentration > 100 µg/g with at least a fivefold increase from baseline. Remission was defined by a FC concentration of ≤100 µg/g and CRP concentration <5 mg/l or a FC concentration <100 µg/g and CRP concentration <10 mg/l and HBI ≤ 4. Patients being in remission at baseline and developing an exacerbation or maintaining remission during follow-up were eligible for further analyses. Healthy subjects (HC) participated in the Maastricht IBS clinical cohort [33].

Fecal samples were collected from all CD and HC subjects at two time points. The CD group comprised a subgroup of 22 patients with sampling at time of remission and subsequent exacerbation (*i.e.* the remission-active or RA group), and 35 patients with two subsequent samples while maintaining remission (*i.e.* RR group). Complete defecations were collected by the study participants, kept at room temperature and brought to the hospital within 12 hours after defecation. Part of the fecal sample of CD patients was sent to the laboratory of Clinical Chemistry for routine analysis of FC and the remaining part was aliquoted and immediately frozen at -80°C for microbiota analysis. Furthermore, blood was collected for routine analysis of CRP.

The standardized computer registration of the IBDSL and the Maastricht IBS cohort (for HC subjects) were used to retrieve baseline demographics, data on disease phenotype, surgery (including (hemi)colectomy and ileocecal resection), medication use and clinical activity scores.

Ethical statement

All study subjects gave written informed consent prior to participation. Both studies have been approved by the Medical Ethics Committee of Maastricht University Medical Center+ and are executed according to the revised declaration of Helsinki (59th general assembly of WMA, Seoul, South Korea, Oct. 2008). The IBD and IBS cohort studies have been registered in the US National Library of Medicine (http://www.clinicaltrials.gov, NCT02130349 and NCT00775060, respectively).

DNA isolation and sequencing

DNA isolation of fecal samples was performed in batches of 11 or 23 by repeated bead beating in combination with the PSP spin stool kit (Stratec Molecular, Berlin, Germany) as described previously [34]. For each DNA isolation batch, one additional isolation was performed on PCR-grade water as a negative control. Amplicon library preparation and sequencing was performed according to a previous published protocol [35]. The 515f/806r primer pair was used to amplify the V4 region of the 16S rRNA gene. PCR reactions were performed using 25 µl NEB Phusion High-Fidelity Master Mix (New England Biolabs, Ipswich, USA), 4 µl 515f/806r primer mix and 30 ng metagenomics DNA under the following conditions: denaturation at 98°C for 3 minutes, followed by 30 cycles of denaturation at 98°C for 45 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 45 seconds. The final elongation step was at 72°C for 7 minutes. Amplicons were purified using the AMPure XP purification (Agencourt, Massachusetts, USA) according to the manufacturer's instructions. Amplicons were mixed in equimolar concentrations and sequenced on an Illumina MiSeq instrument.

Data analyses

Sequencing data were processed and analyzed by QIIME 1.9 (quantitative insights into microbial ecology) according to previous published protocols with some modifications [30,36]. Sequences were paired-end joined by using fastq join and filtered using default values except for the phredscore (Q>19). Sequences were clustered into operational taxonomic units (OTUs) based on an identity score of 97% using UCLUST and clustered against the Green genes reference database version 13.8. OTUs exceeding more than 3% dissimilarity were discarded and were not included into downstream analyses. The OTU table was rarefied to 9,208 seq/sample. OTUs present in at least 10% of all samples were used for downstream analyses.

Differences in phyla abundances between the first and second time-point was analyzed by means of the Wilcoxon signed rank test.

The following alpha diversity indices were calculated to examine the microbial richness and diversity within samples: observed species, Chao1, Shannon index and PD whole tree. To analyze the changes in the microbial richness and diversity between the first and second visit, the differences in alpha diversity indices between the two time-points were calculated per individual and average differences were subsequently compared between the study groups using the Mann-Whitney U test.

The Bray-Curtis dissimilarity and (un)weighted Unifrac distance within subjects was determined to investigate both the changes in the microbiota community structure within subjects over time as well as to determine the dissimilarity in the microbiota community composition between subjects [37]. The beta-diversity within subjects was compared between the three study groups

(healthy individuals, CD patients staying in remission and CD patients in remission and subsequent exacerbation) by means of the Mann-Whitney U test. Principal coordinate analysis (PCoA) was used to visualize the microbiota structure between the different groups.

RESULTS

Study population

A total of 144 fecal samples of 57 CD patients and 15 healthy individuals were collected in this study. Baseline characteristics of all study subjects are presented in table 3.1. Healthy individuals were generally younger at inclusion as compared to CD patients. In the RR group, 70 fecal samples of 35 CD patients were collected during remission with a median (IQR) time interval of 14 (11-21) weeks between the two sampling moments. In the RA group, 44 samples of 22 CD patients were collected during remission and subsequent exacerbation with a time interval of 20 (8-36) weeks between the two sampling moments. Each healthy individual collected two fecal samples with a time interval of 13 (12-16) weeks between the sampling moments. Neither any apparent difference was found in medication use between the fecal samples of the different CD patient populations, nor within each patient group over time (table 3.2). Five RR and four RA patients had a medication change between consecutive samples during the study period. In the RR group, prednisone was stopped in 1 patient, mesalazine in 1 patient and biologicals in two patients, while one patient started with mesalazine and one other patient started with biologicals. In the RA group, 2 patients started with biologicals, 2 with thiopurines and 1 with mesalazine.

Table 3.1. CD patients characteristics

	Remission-remission (n=35)	Remission-active (n=22)	Healthy controls (n=15)
Age at inclusion (median, IQR)	43 (33-53)	38 (26-59)	25 (23-30)
Male (%)	10 (28.6)	10 (45.5)	8 (53.3)
Smoking (%)	8 (22.9)	2 (9.1)	0 (0)
Age at diagnosis[1] (%)			
A1 (<16 year)	1 (2.9)	1 (4.5)	Na
A2 (17-40 year)	30 (85.7)	13 (59.1)	
A3 (>40 year)	4 (11.4)	8 (36.4)	
Disease localization[1] (%)			
L1 (ileal)	12 (34.3)	7 (31.8)	Na
L2 (colonic)	8 (22.9)	7 (31.8)	
L3 (ileocolonic)	15 (42.9)	8 (36.4)	
Phenotype at inclusion[1] (%)			
B1 (nonstricturing, non penetrating)	26 (74.3)	12 (54.5)	Na
B2 (stricturing)	6 (17.1)	5 (22.7)	
B3 (penetrating)	3 (8.6)	5 (22.7)	
Abdominal Surgery[2]	8 (22.9)	4 (18.2)	0 (0)

[1] According to Montreal Classification
[2] includes (hemi)colectomy and ileocecal resection

Table 3.2. Medication use and time between sampling moments for active and remission samples

	RR		RA	
	Remission (n=35)	**Remission (n=35)**	**Remission (n=22)**	**Active (n=22)**
Medication (%)				
Mesalazine	5 (14.3)	5 (14.3)	4 (18.2)	5 (22.7)
Thiopurines	11 (31.4)	11 (31.4)	7 (31.8)	9 (40.9)
Biologicals	18 (51.4)	17 (48.6)	13 (59.1)	15 (68.2)
Prednisone	1 (2.9)	0 (0)	1 (4.5)	1 (4.5)
PPI	7 (20)	7 (20)	8 (36.4)	8 (36.4)
Antibiotics[#]	1 (2.9)	0 (0)	1 (4.5)	0 (0)
Time between sampling moments (week, median, IQR)		14 (11-21)[1]		20 (8-36)[2]

[1] Time between first remission and second remission samples
[2] Time between first remission and first active samples
[#] Ciprofloxacin and cotrimoxazol were used two and one month, respectively prior to sample collection

Sequencing

Joining of the forward and reverse illumina reads resulted in 4,405,908 sequences. After quality filtering and binning, a total of 3,122,584 sequences with an average of 20,277 sequences per sample (range: 9,208-29,062 sequences/sample), corresponding to 1,829 OTUs, were available for downstream analysis.

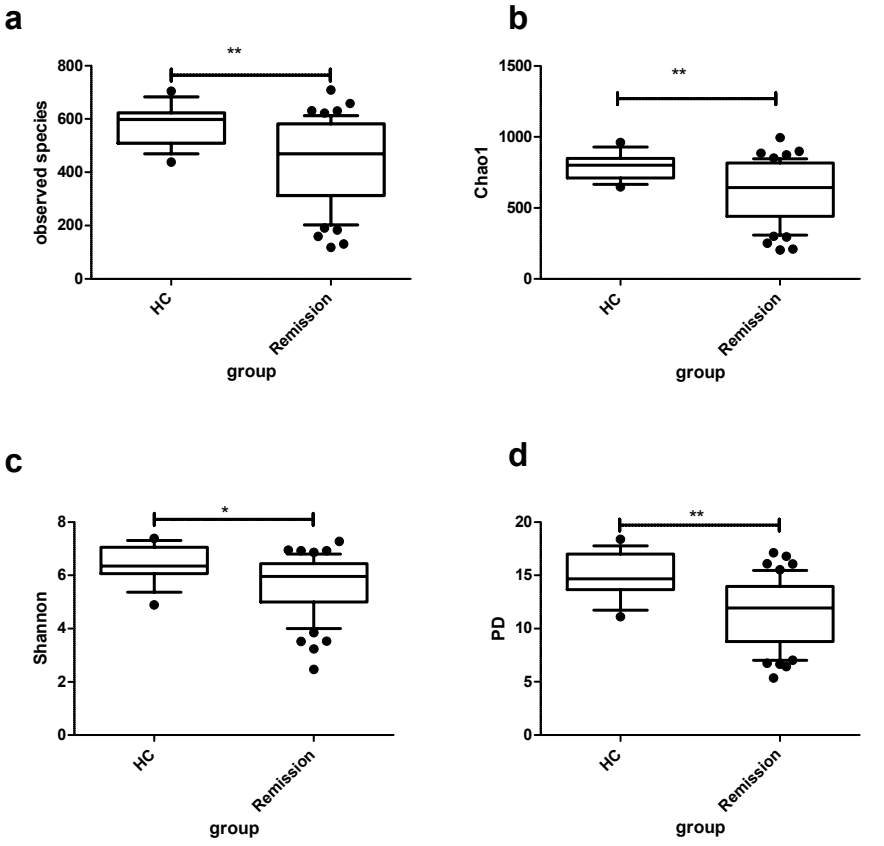
The fecal microbiota composition was dominated by the phyla Firmicutes and Bacteroidetes (baseline median (IQR) relative abundance) in healthy individuals (0.47 (0.41-0.56) and 0.46 (0.34-0.51), respectively), CD patients maintaining remission (0.42 (0.26-0.53) and 0.49 (0.36-0.58), respectively) and CD patients in remission following an exacerbation (0.52 (0.38-0.61) and 0.36 (0.28-0.46), respectively).

Microbial richness and diversity

At baseline, CD patients had a significantly lower fecal microbial richness and diversity as compared to healthy individuals as indicated by the number of observed species (median(IQR): 469.5 (312.9-581.9) and 598.2 (509.2-623.3), respectively; p=0.002), Chao1 index (median (IQR): 644.7 (440.1-817.2) and 800.4 (711.0-849.7), respectively; p=0.004), Shannon index (median (IQR): 6.0 (5.0-6.4) and 6.3 (6.1-7.1), respectively; p=0.014) and PD whole tree (median (IQR): 12.0 (8.8-14.0) and 14.7 (13.7-17.0), respectively; p=0.001). A subgroup of CD patients at baseline seems to have similar fecal microbial richness and diversity as the healthy group, while others showed an explicit lower microbial richness and diversity as compared to the healthy group (figure 3.1).

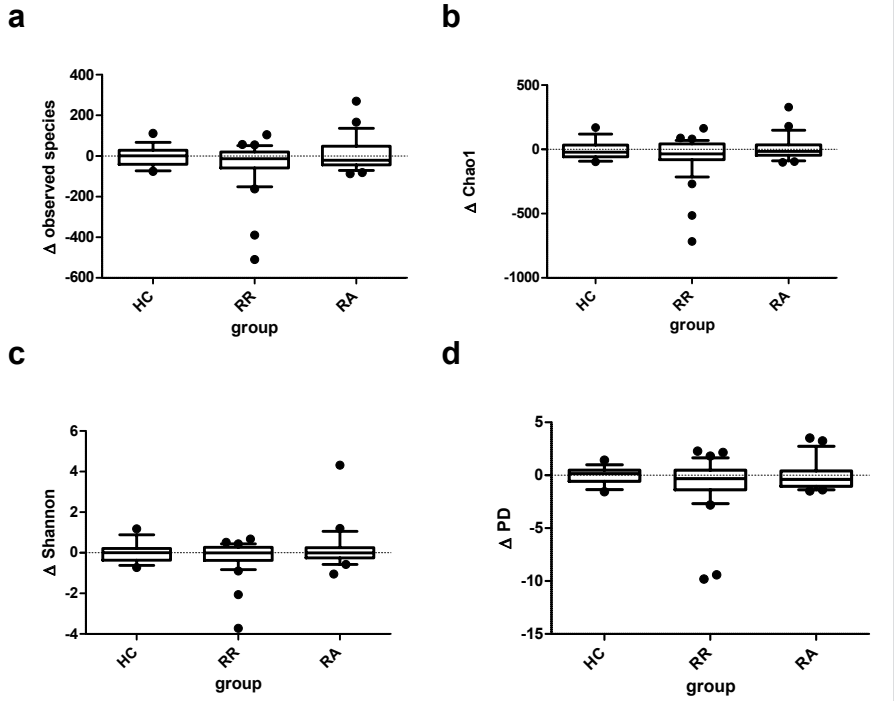
The changes in alpha-diversity indices within study subjects during the study period were compared between CD patients maintaining remission, CD patients in remission followed by an exacerbation and healthy individuals. No significant differences were found between the three study groups for the changes in number of observed species, Chao1 index, Shannon index and PD whole tree (figure 3.2).

Figure 3.1. Alpha diversity indices (observed species (a), Chao1 (b), Shannon index (c) and PD whole tree(d)) of healthy controls (HC1) and Crohn's disease patients at baseline (T1).



All patients were in remission at baseline. * and ** indicates $p<0.05$ and $p<0.01$ respectively.

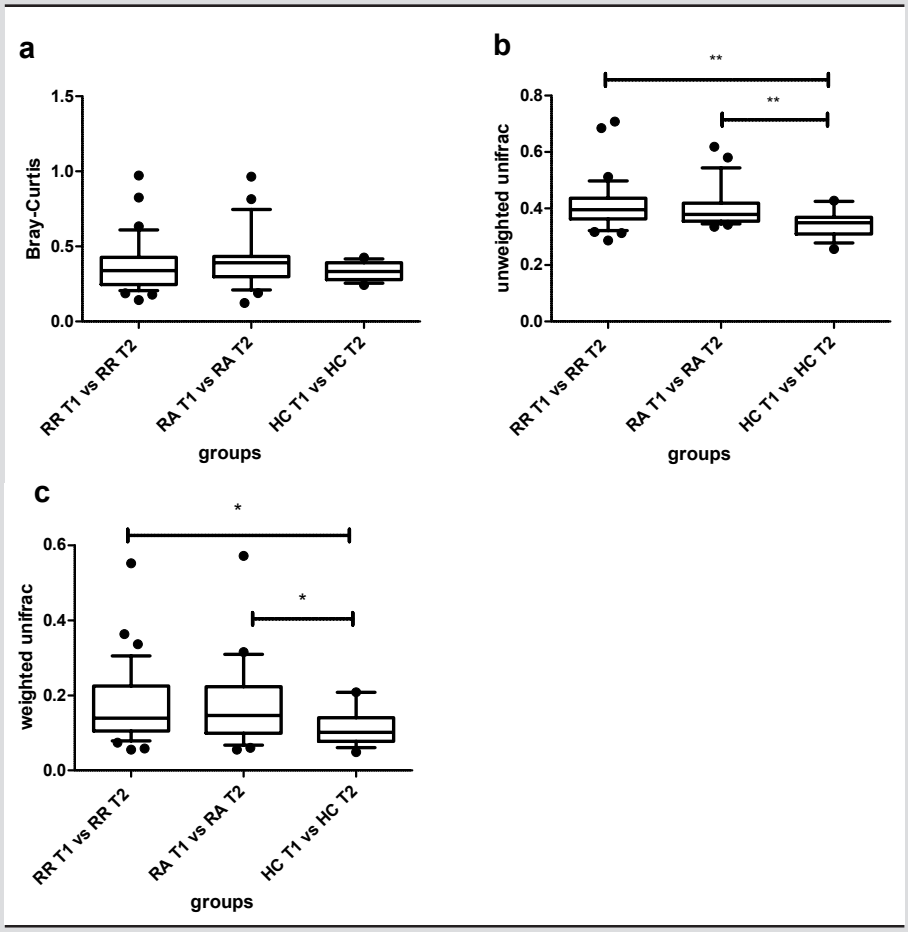
Figure 3.2. Changes in alpha-diversity indices between first and second time-point (Δ of alpha-diversity metric) of healthy individuals (HC), CD patients staying in remission (RR) and CD patients in remission followed by an exacerbation (RA).



Microbial community structure

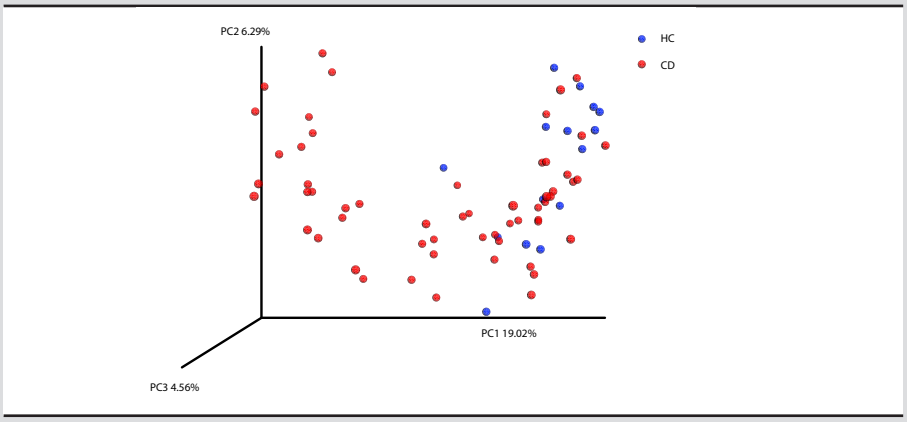
Changes in the fecal microbial community structure between the first and second sample within individuals were assessed by means of the Bray-Curtis and (un)weighted Unifrac. The microbiota of CD patients appeared to be less stable than the microbiota of HC as indicated by the significantly larger within-subject (un)weighted Unifrac distances (figure 3.3). However, no differences in microbiota stability were observed when comparing CD patients maintaining remission versus those who developed an exacerbation (figure 3.3). These results indicate that the fecal microbiota of healthy individuals is more stable than that of CD patients and that the fecal microbiota stability of CD patients was not affected by developing an exacerbation.

Figure 3.3. Within-subject beta-diversity in healthy individuals (HC T1 vs HC T2), CD patients staying in remission (RR T1 vs RR T2) and CD patients in remission and subsequent exacerbation (RA T1 vs RA T2).



A PCoA was performed based on the unweighted Unifrac distance of the fecal microbiota of CD patients and healthy individuals at baseline and did not show a clear separation between the two populations (figure 3.4). Again, a subset of the CD patients appeared to cluster apart from the healthy individuals.

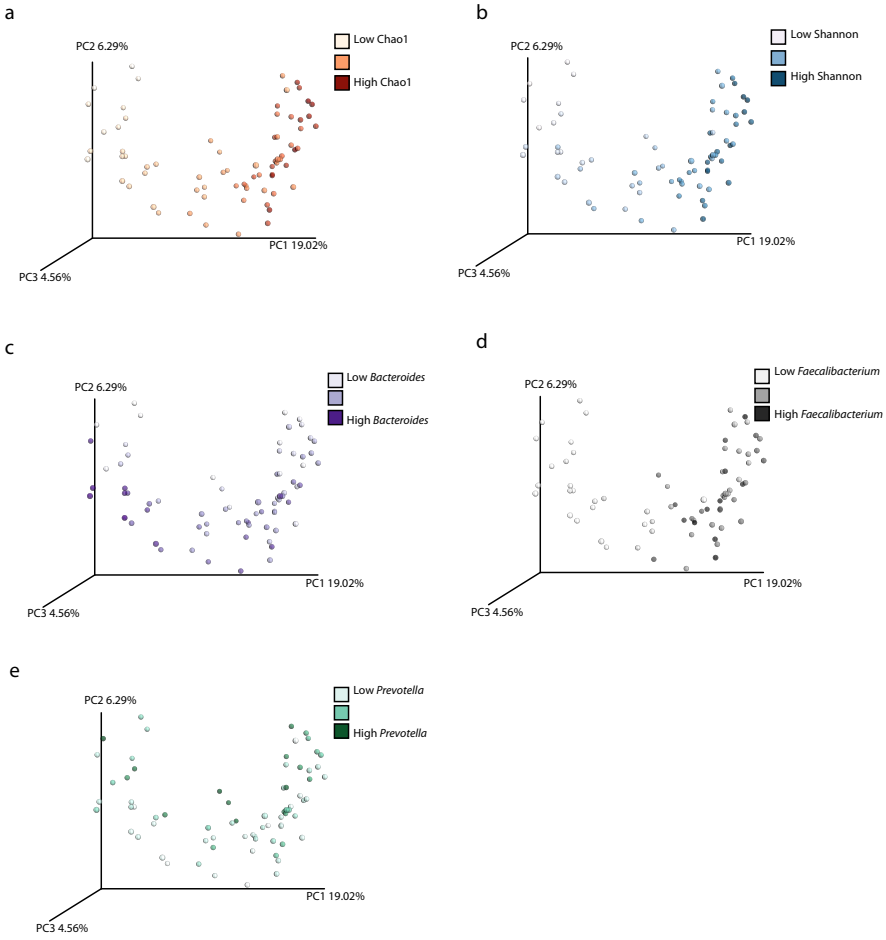
Figure 3.4. PCoA based on the unweighted Unifrac distance metric of fecal microbiota of healthy controls (blue) and Crohn's disease (red) patients at baseline (T1).



We subsequently examined the factors driving the separation of this subset of CD patients and found that the relative abundance of *Faecalibacterium spp.* and the microbial richness and diversity were driving the separation along PC1 (figure 3.5a,b,d). Based on this observation, the subgroup of CD patients that are more deviant from the HC fecal microbiota, seem to be characterized by a lower microbial richness and diversity and a lower relative abundance of *Faecalibacterium spp*.

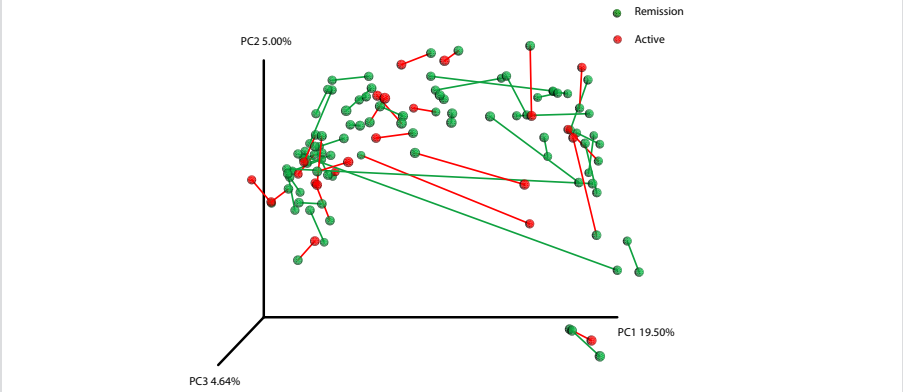
A second PCoA was performed based on the unweighted Unifrac distance of fecal samples from CD patients at baseline (remission) and the second time-point (remission or exacerbation) (figure 3.6). The distance between the two consecutive samples of the same individual is indicated by the green (RR) or red (RA) vector. No discrete separation between remission and active samples is observed. Furthermore, no consistent pattern in the PCoA is observed with respect to the movements of the samples at baseline and follow-up. Finally, no clustering based on medication use or medication change during the study period (mesalazines, thiopurines, biologicals, prednisone and PPI) was found (supplemental table S3.1, supplemental figure S3.1 and supplemental figure S3.2).

Figure 3.5. PCoA based on unweighted Unifrac distance metric of fecal microbiota of healthy controls and Crohn's disease patients at baseline (T1).



Samples are colored based on the a) Chao1 (white-red), b) Shannon (white-blue), relative abundance of c) *Bacteroides spp.*,(white-purple), d) *Faecalibacterium spp.* (white-black) and e) *Prevotella spp.* (white-green).

Figure 3.6. PcoA based on unweighted Unifrac distance metric of fecal microbiota of CD patients at baseline (T1) and at second sampling time-point (T2).



Remission samples are indicated in green and active samples are indicated in red. Vectors between samples indicate the distance between two consecutive samples of the same individual. Green vectors indicate two consecutive samples with concordant disease status (remission) and red vectors indicate two consecutive samples with disconcordant disease status (remission to active)

DISCUSSION

To the best of our knowledge, this is the largest longitudinal study that comprehensively investigated the stability of the fecal microbiota of adult CD patients during their disease course. First, CD patients showed a lower microbial richness and diversity when compared to healthy controls. Second, this did not change over time and was not affected by differences in disease course. Third, a subset of CD patients clustered apart from healthy controls and were characterized by a low microbial diversity and relative low abundance of *Faecalibacterium spp.*

By collecting multiple samples of healthy individuals and CD patients both with and without a changing disease activity over time, we were able to assess the microbial stability and to investigate the microbial changes during remission and active disease, thereby avoiding potential confounding associated with cross-sectional studies. The present study confirms previous observations that the fecal microbiota of CD patients is less diverse as compared to healthy individuals [38]. We also demonstrated that healthy individuals have a stronger temporal stability of the microbial community structure when compared to CD patients, regardless whether these patients maintained remission or developed an exacerbation. This observation is consistent with previous results of Scanlan *et al* who demonstrated by means of PCR-DGGE that the healthy microbiota composition was more stable over time as compared to the microbiota of CD patients [39].

The magnitude of changes in microbial richness and diversity over time were similar between healthy individuals and CD patients, and did not differ between patients staying in remission compared to those developing an exacerbation. The absence of an altered stability in patients developing active disease as compared to patients maintaining remission is in contrast with several cross-sectional studies showing differences in microbiota members between CD patients in active and quiescent disease [17,40], and questions the involvement of the overall microbiota in the development of an exacerbation. This may be affected by confounding factors, therefore we investigated the role of disease phenotype, medication use and surgery. We did not find clustering of samples based on disease location or medication use (mesalazines, thiopurine, biologicals, prednisone and PPI), abdominal surgery, age at diagnosis, disease phenotype at inclusion. Still, small shifts in specific taxa may be present in CD patients with changing disease activity over time. Two previous small studies demonstrated shifts in the microbiota composition when patients developed an exacerbation, but these shifts were patient specific and no general bacterial signatures were found for all patients with exacerbation [25,39]. Although we included more patients than the previous two studies, neither did we observe a consistent pattern in the changes in the microbiota community structure of CD patients with changing or stable disease course over time. It is plausible that patient specific changes are present, but due to our focus on the microbiota composition, these changes remained undetected. Therefore,

further analyses focusing on (small) changes in individual taxa are warranted.

CD can be categorized in different phenotypes based on characteristics such as disease localization and behavior (*i.e.* structuring and/or penetrating behavior). Our results indicate a subgroup of CD patients of whom the microbiota composition deviated from the microbiota of healthy controls, characterized by a low microbial richness and diversity and a low relative abundance of *Faecalibacterium.* Subgroups of CD patients that clustered apart from other CD patients and healthy controls have also been demonstrated in previous studies [8,41,42]. Lewis *et al* studied the effect of treatment (TNF inhibitors, exclusive enteral nutrition or partial enteral nutrition with ad libitum diet on pediatric CD patients) and found a subgroup of active patients that clustered apart from healthy individuals, amongst others due to a low relative abundance of *Faecalibacterium* [41]. These observations suggest the existence of a subgroup of CD patients with a different microbiota composition which could be explained by factors such as disease phenotype, localization or medication use. We could not find clear differences in disease phenotype, disease localization or medication use/change when comparing the CD patients with a more deviant versus those with a more 'healthy' microbiota profile, but numbers were relatively small. Larger studies are needed to further characterize the subgroup of CD patients that did not cluster with healthy individuals, and to investigate whether this deviant microbiota has an impact on disease phenotype or disease course.

Our study has several strengths, including the longitudinal study design. Although cross-sectional microbiota studies are restricted by the large inter-individual variation of the microbiota, most microbiota studies on disease activity in adult CD patients are based on a cross-sectional design. Longitudinal studies are able to circumvent this limitation. To our knowledge, ten other prospective studies were published investigating the microbiota and CD activity over time. Three were performed in pediatric patients [13,41,43] and four in adult patients focusing on treatment response [23,24] or recurrence after colon resection [27,28]. Our study is so far the largest study that comprehensively examines the microbiota in relation to the natural disease course in CD patients.

As repeated endoscopy, which is the golden standard for disease activity assessment, is too invasive in a patient cohort from daily clinical practice, most studies use clinical activity indices to assess disease activity [13,19,23,24,39]. Since the association of clinical activity indices with mucosal inflammation based on endoscopic scores is only moderate [44] we used fecal calprotectin levels to indicate an exacerbation in this study. A combination of clinical and inflammation markers, being accepted surrogate markers for mucosal inflammation, was used to define remission [45,46].

In conclusion, our prospective study showed that the microbiota of CD patients is less stable over time as compared to healthy individuals, but the temporal microbiota stability in CD patients was not affected by the development of an exacerbation. Furthermore, an altered microbiota composition, characterized by a low bacterial diversity and low abundance of *F. prausnitzii,* seems to be present in subgroups of CD patients, while the remaining CD patients cluster with healthy individuals, which warrants further investigation.

FUNDING

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SUPPLEMENTAL INFORMATION

Supplemental table S3.1. Subjects characteristics of CD patients with a low and high unweighted Unifrac distance

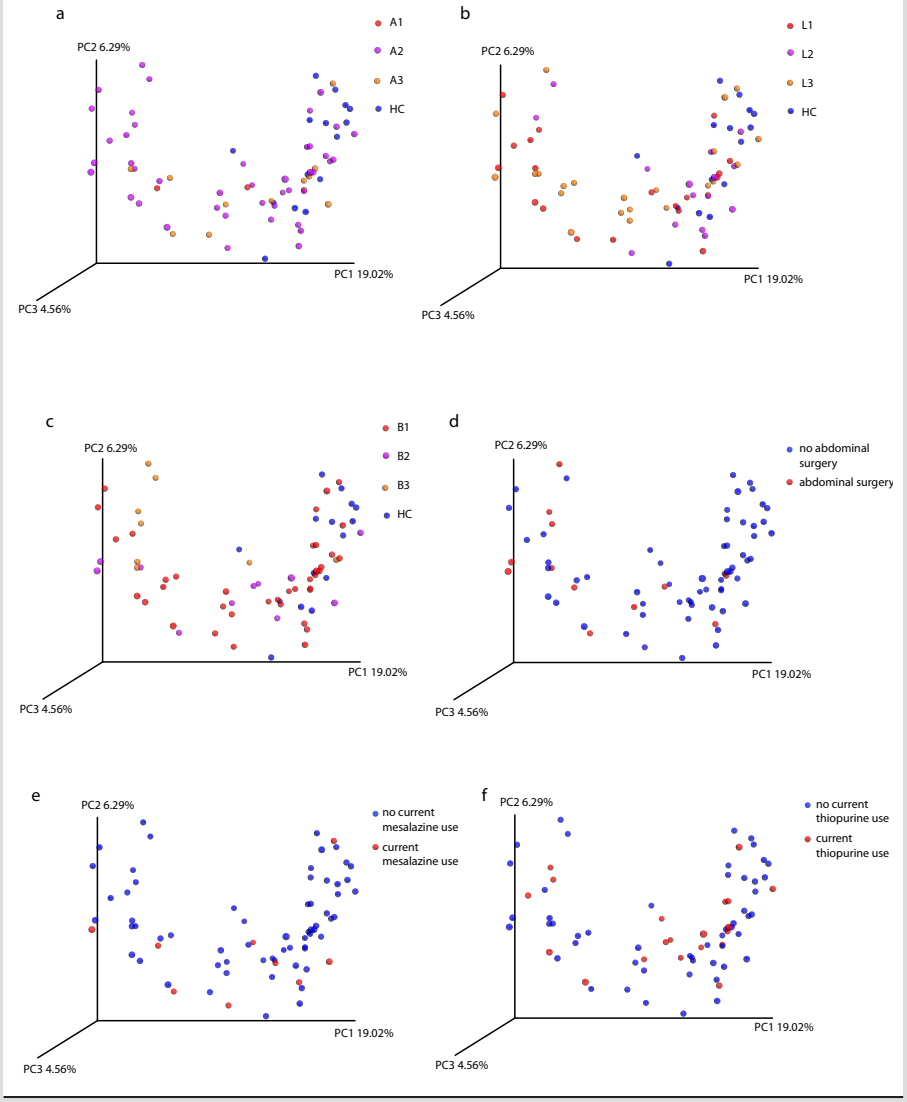
	Unweighted Unifrac ≤0.4152275 (n=38)[1]	Unweighted Unifrac >0.4152275 (n=19)[2]
Age at inclusion (median, IQR)	43 (29-57)	43 (33-50)
Male (%)*	17 (44.7)	3 (15.8)
Smoking (%)	5 (13.2)	5 (26.3)
Age at diagnosis (%)		
A1	1 (2.6)	1 (5.3)
A2	26 (68.4)	17 (89.5)
A3	11 (28.9)	1 (5.3)
Disease localization (%)		
L1	13 (34.2)	6 (31.6)
L2	10 (26.3)	5 (26.3)
L3	15 (39.5)	8 (42.1)
Phenotype at inclusion (%)		
B1	25 (65.8)	13 (68.4)
B2	9 (23.7)	2 (10.5)
B3	4 (10.5)	4 (21.1)
Abdominal surgery (%)	6 (15.8)	6 (31.6)
Medication (%)		
Mesalazine	7 (18.4)	2 (10.5)
Thiopurine	13 (34.2)	7 (36.8)
Biologicals	18 (47.4)	13 (68.4)
Prednisone	0 (0)	2 (10.5)
PPI	10 (26.3)	5 (26.3)
Antibiotics current	0 (0)	0 (0)
Antibiotics past	0 (0)	2 (0)

* p=0.028

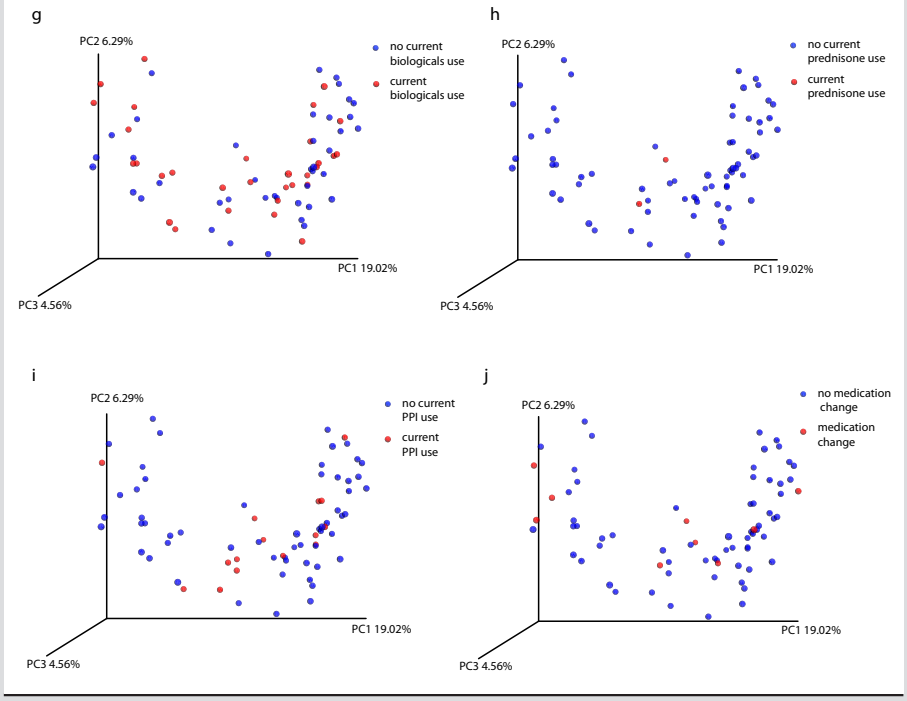
[1] 5/9 patients had a medication change during the study period.

[2] 4/9 patients had a medication change during the study period.

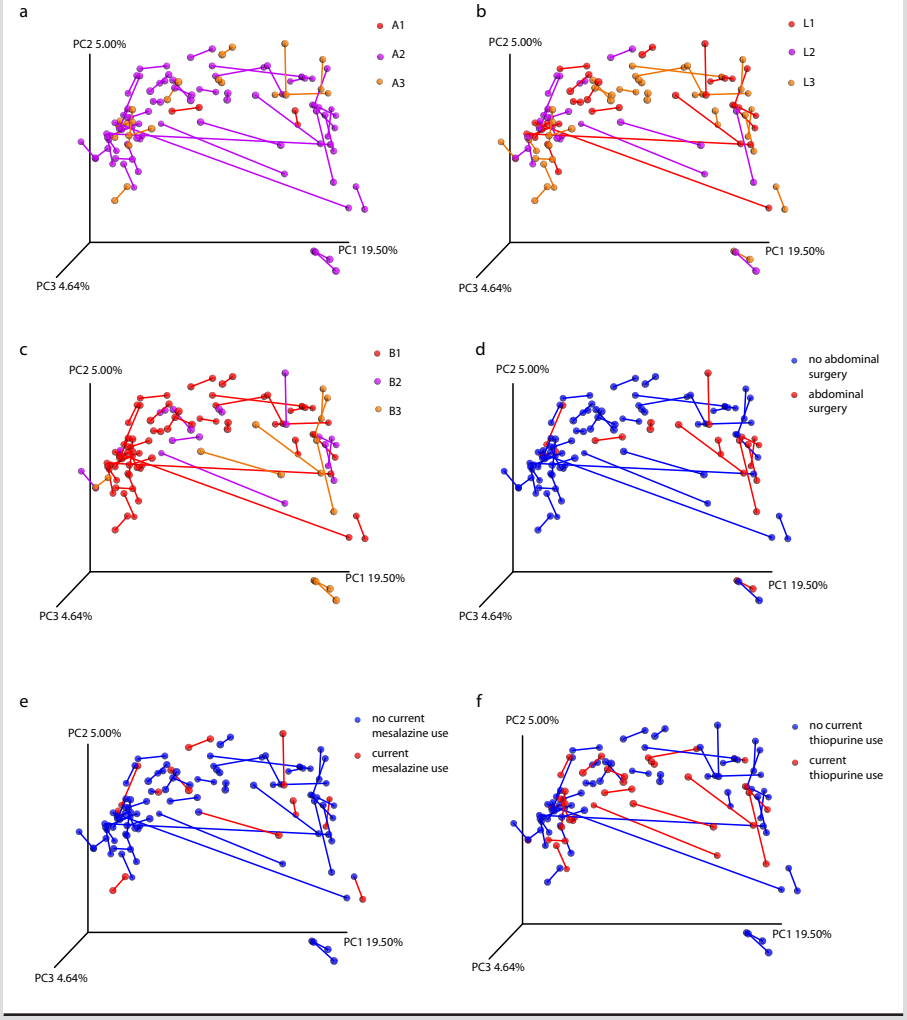
S3.1. Principal coordinate analysis based on unweighted UniFrac distance for healthy subjects and CD patients at baseline (T1) colored based on age at diagnosis (a), disease localization (b), disease phenotype at inclusion (c), abdominal surgery (d), mesalazines use (e), thiopurine use (f), biologicals use (g), prednisone use (h), PPI use (i) and medication change (j).



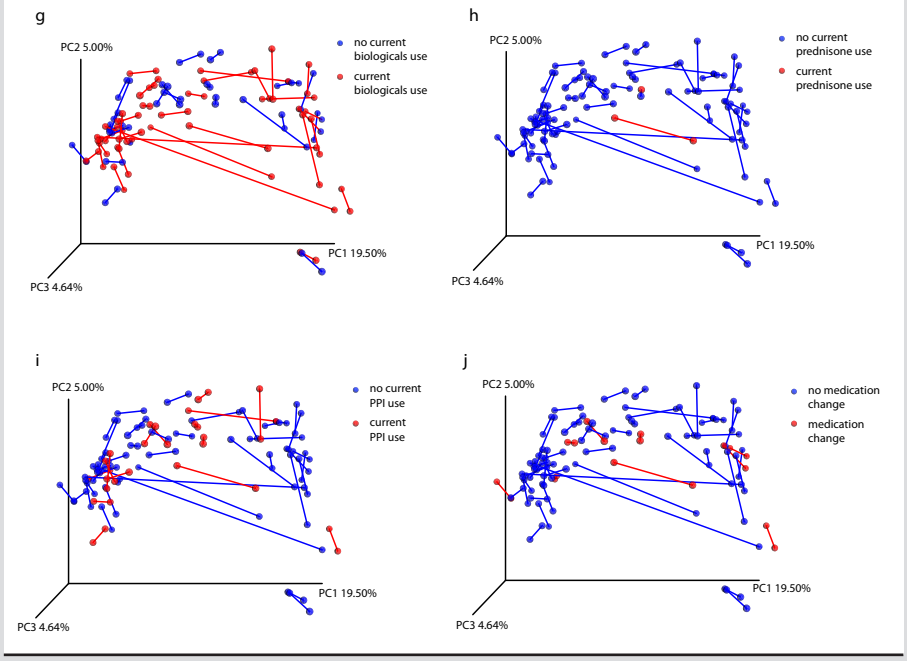
S3.1. Continued.



S3.2. Principal coordinate analysis based on unweighted UniFrac distance for CD patients at baseline (T1) and at second sampling time-point (T2) colored based on age at diagnosis (a), disease localization (b), disease phenotype at inclusion (c), abdominal surgery (d), mesalazines use (e), thiopurine use (f), biologicals use (g), prednisone use (h), PPI use (i) and medication change (j).



S3.2. Continued.



4

The fecal microbiota as a biomarker for disease activity in Crohn's disease

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ABSTRACT

Monitoring mucosal inflammation is crucial to prevent complications and disease progression in Crohn's disease (CD). Endoscopy is the current standard, but is invasive. Clinical activity scores and non-invasive biochemical markers do not correlate well with mucosal inflammation. Microbial perturbations have been associated with disease activity in CD. Therefore, we aimed to investigate its potential use to differentiate CD patients in remission from those with an exacerbation. From 71 CD patients repeated fecal samples were collected, resulting in 97 active disease and 97 remission samples based on a combination of biochemical and clinical parameters. The microbiota composition was assessed by pyrosequencing of the 16S rRNA V1-V3 region. Random Forest analysis was used to find the most discriminatory panel of operational taxonomic units (OTUs) between active and remission samples. An independent internal validation set was used to validate the model. A combination of 50 OTUs was able to correctly predict 73% of remission and 79% of active samples with an AUC of 0.82 (sensitivity: 0.79, specificity: 0.73). This study demonstrates that fecal microbial profiles can be used to differentiate between active and remission CD and underline the potential of the fecal microbiota as a non-invasive tool to monitor disease activity in CD.

INTRODUCTION

In the past decades, the incidence of Inflammatory bowel diseases (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), has been increasing in industrialized countries in Europe and North America. Currently, a rise has also been reported in Asian countries, in line with westernization [1,2]. UC is characterized by continuous mucosal inflammation in the colon, while CD can affect any part of the gastrointestinal tract and can be transmural and discontinuous. Both UC and CD are associated with periods of active inflammation with symptoms such as abdominal pain and (bloody) diarrhea, alternated with periods of remission [3]. Treatment is merely symptom-based and focuses on inducing or maintaining remission. However, current treatment modalities are associated with mild to severe side effects and limited long-term efficacy [4,5]. Thereby, IBD has a significant impact on the patient's quality of life and accounts for substantial costs to the health care system, especially during exacerbations [6].

Monitoring mucosal inflammation is crucial to limit disease progression and complications. Endoscopy is the current standard, but is an expensive and invasive procedure with risk of complications [7]. Clinical activity scores, such as the Harvey-Bradshaw index (HBI) for CD and the simple clinical colitis activity index (SCCAI) for UC, are often used in clinical practice and therapeutic intervention trials, but do not correlate well with mucosal inflammation [8]. In daily clinical practice, inflammatory markers such as C-reactive protein (CRP) and fecal calprotectin (FC) are often used to evaluate disease activity. CRP, however, is not specific for intestinal inflammation [9,10]. FC correlates well with endoscopic scores in UC, but its' diagnostic accuracy is less for CD due to a limited sensitivity for the proximal colon and small bowel [9,10]. Therefore, new non-invasive markers for active disease are needed, especially for patients with CD.

Current biochemical markers used to monitor disease activity, are often non-specific and not associated with possible pathophysiological mechanisms. Nowadays, it is generally accepted that the microbiota plays an important role in the development and disease progression of IBD [11,12]. According to previous studies the microbiota composition of CD patients is characterized by a decrease of fecal and mucosal microbial diversity and a change in the relative abundance of specific bacterial taxa (*e.g.* reduction of *Faecalibacterium prausnitzii*) compared to the microbiota of healthy individuals [13–15]. Furthermore, also clear differences have been reported in active versus quiescent disease, although results between studies are inconsistent, most likely due to methodological differences [16–25]. A study by Swidsinski *et al* showed that concentrations of mucosal associated bacteria increased with disease severity [26]. Moreover, antibiotics are able to induce remission in active CD patients and are effective against anal lesions and in the prevention of post-operative recurrence CD [27]. These studies suggest that the microbiota plays an important role in inducing exacerbations.

Possible differences in the microbiota composition related to disease activity may result in markers for disease monitoring. So far, specific bacterial taxa clearly associated with disease activity have not been identified yet. Investigating the microbial community structure (*i.e.* combinations of OTUs) rather than specific microbial taxa might be more effective in investigating the role of the intestinal microbiota in IBD, as previous studies have demonstrated [28,29]. Papa *et al* was able to distinguish paediatric IBD patients in remission and during an exacerbation as defined by clinical indices with an AUC of 0.72 based on the fecal microbiota composition [28]. However, it was previously shown that CD and UC patients have a different microbiota structure and by collating CD and UC patients together, the classification might not be optimal [30,31]. A second study in paediatric CD patients, was able to predict an exacerbation within six months after diagnosis based on the fecal microbiota with an accuracy of 67% [29]. Studies using the fecal microbiota to predict disease activity in adults are lacking. Therefore, the aim of the present study was to investigate the potential use of microbiota profiling to accurately differentiate between Crohn's disease patients in remission from those with an exacerbation.

MATERIAL AND METHODS

Study population

A total of 194 fecal samples (97 remission, 97 active) from 71 CD patients were included in this study. IBD was diagnosed based on clinical and endoscopic or radiological findings conform the ECCO guidelines [32]. These patients were part of a prospective follow-up cohort of IBD outpatients of the population-based IBDSL cohort [33,34]. Clinical data, blood and feces were collected at each visit to the outpatient clinic and during an exacerbation. Fecal samples were collected by the patients at home and brought to the hospital within 24 hours after defecation. Upon arrival, part of the sample was sent to the laboratory of Clinical Chemistry for routine analysis of CRP and FC. The remaining part was aliquoted and frozen directly at -80°C for microbiota analyses. For the purpose of the present study, fecal samples collected within 1 month after a course of antibiotics were excluded.

Baseline demographics, data on disease phenotype, medication use and clinical activity scores were retrieved using the standardized computer registration of the IBDSL cohort [33]. Disease activity was defined by the Harvey Bradshaw index (HBI) in combination with serum CRP or FC.[34] Active disease was defined by a FC>250 µg/g [35]. Remission was defined by a HBI≤4 in combination with both serum CRP <5 mg/l and FC<100 µg/g.

Ethical statement

The patients included in the present study gave written informed consent prior to participation. The study has been approved by the Medical Ethics Committee of Maastricht University Medical Center+ and is executed according to the revised declaration of Helsinki (59th general assembly of WMA, Seoul, South Korea, Oct. 2008). The study has been registered in the Central Committee on Research Involving Human Subjects (CCMO) registry under file number NL24572.018.08.

DNA isolation of fecal samples

Frozen aliquots of fecal samples were cut on ice to prevent thawing of the fecal samples and approximately 200 mg was added to vials containing PSP lysis buffer (Stratec Molecular, Berlin, Germany), 0.5 g of 0.1 mm zirconia/silica beads and 4 glass beads of 3.0-3.5 mm (BioSpec, Bartlesville, USA). The fecal samples were homogenized in a MagNALyser instrument (Roche, Basel, Switzerland) in three cycles of 1 min at a speed of 5500 rpm. Samples were kept on ice for one minute in between cycles. DNA isolation was continued using the PSP Spin Stool Kit (Stratec Molecular, Berlin, Germany) according to the manufacturers' instructions. DNA was finally eluted in 200 µl TE-buffer. Negative control samples (PCR grade water) were included in each batch of samples for DNA-isolation, and handled in exactly the same way as the fecal samples, in order to rule out contamination during the isolation procedure.

454 pyrosequencing

Amplification of the V1-V3 16S rRNA amplicons was performed using forward primers consisting of a 9:1 ratio mixture of 8F and 8F-Bif, respectively, and reverse primer 534R as described previously [24]. The PCR reaction was performed using 1x FastStart High Fidelity Reaction Buffer, 1.8 mM $MgCl_2$, 1mM dNTP solution, 5 U FastStart High Fidelity Blend Polymerase (Roche, Indianapolis, USA), 0.2 μM forward primer, 0.2 μM reverse primer and 1 μl of template DNA (15-50 ng/uL) under the following conditions: denaturation at 94°C for 3 minutes, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 45 seconds and extension at 72°C for 5 minutes. The final elongation step was at 72°C for 10 minutes. Negative controls were included in each PCR run by replacing 1uL DNA by PCR grade water.

The amplicons were purified using AMPure XP purification according to the manufacturer's instructions and eluted in 25 μl TE. Amplicon concentrations were determined by Quant-IT Pico Green dsDNA reagent kit (Invitrogen, New York, USA) using the Victor3 Multilabel Counter (Perkin Elmer, Waltham, USA). Thereafter, amplicons were mixed in equimolar concentrations to establish an equal representation of each sample for the emulsion PCR (emPCR). After emPCR (Titanium emPCR Kit (Lib-L)), pyrosequencing was performed according to the manufacturer's instructions (Roche, Brandford, USA).

Data presentation and statistical analyses

Baseline demographics and disease phenotype at time of inclusion of the CD patients with active disease versus remission are presented as median and range for continuous variables and numbers and percentages for categorical variables.

The V1-V3 16S rDNA bacterial sequences that were used in this paper have been submitted to the European Nucleotide Archive (ENA) under accession PRJEB11845.

The raw pyrosequencing reads were passed through quality filters using Mothur version 1.32.1 to reduce error rates [36]. Sequences with perfect proximal primer fidelity, a minimum average quality score of 25 over a window size of 50 nucleotides, a read length between 200 and 590, a maximum of one ambiguous base call and a maximum homopolymer length of 6, were retained for further analyses. Sequences were de-multiplexed and clustered by UCLUST algorithm into operational taxonomic units (OTUs) based on 97% similarity against the Greengenes reference set version August 2013 in Qiime 1.8. [37]. Default parameters for UCLUST were applied apart for the following parameters: maxrejects=100 and stepwords=16. Sequences that did not cluster to reference sequences were discarded to reduce the influence of sequencing errors. To control for variation in sequencing effort the OTU-table was subsequently rarefied to 4,930 sequences/sample.

Random Forest (RF) analysis was used to find the most discriminatory OTUs between CD patients with active disease versus remission. As it is unlikely that an OTU present in a minority of samples will have group-related importance, OTUs were only included in the statistical analysis if they were detected in at least 20% of the samples in one of the groups. Prior to actual RF analyses, the microbiome data were transformed via an inverse hyperbolic sine transformation and then mean centered per individual patient [38]. The first step accounts for skewness and can deal with sparse microbiome data. The mean centering per individual diminishes the influence of inter-individual variation.

In the current study, two different RF models were built. The first RF model (with 700 trees), based on 90 different randomly selected subsets, aimed to find the most discriminatory OTUs between active CD and CD in remission. The second RF model was performed to demonstrate the contribution of the most discriminatory OTUs in differentiating active and inactive CD and to test the classification performance of the model in the validation set. The second RF model (with 700 trees) was based on 300 randomly selected subsets. For both RF models, each subset contained all samples from the same individual either in the training set, consisting of 80% of all samples, or in the validation set (the remaining 20%). Thereby, the RF classification model was never trained on part of the measurements of one subject and tested on the remaining measurements of that subject.

The final classification of each sample was determined by a majority of votes (>50%) from 300 RF classification models. The final performance of the RF classification model is demonstrated by the receiver operating characteristic (ROC) curve.

After tree construction, RF computes the proximities, which indicate the similarity between samples. The proximities obtained from the second RF analyses, were used to visualize the differences between the two groups (active or remission) by Principal Component Analysis (PCA). A canonical correlation analysis (CCA) was performed to check whether the selected OTUs correlated with FC concentrations as indicator of intestinal inflammation.

The directions of bacteria changes in CD patients in active and remission was investigated via boxplot analysis, where the distributional characteristics and the count of bacteria can be shown [39].

The potential confounding effect of medication use at the time of sampling (*i.e.* use of biologicals (anti-TNF), mesalazines and thiopurines), disease location (ileal (L1), colonic (L2), ileocolonic (L3)) according to the Montreal classification), colectomies and age at time of sample collection, on each of the individual 50 OTUs associated with disease activity was tested using the Friedman test with post-hoc correction for multiple testing. To test whether the set of discriminatory

OTUs was statistically influenced by the possible confounding factors (*i.e.* use of medication, disease location, colectomies and age), we used regularized multivariate analysis of variance (rMANOVA)[40] . For age, the patients were grouped into three classes: patients younger than 30, those in age range 30-50 and patients over ≥ 50 years of age. A false discovery rate (FDR) cut off value of 0.05 was used to correct for multiple testing.

All analyses were done in Matlab2014a.

RESULTS

Study population

A total of 194 fecal samples of 71 Crohn's disease patients (18-70 years) were included in this study. Baseline characteristics of the 71 patients are presented in table 1. A single sample was available for 14 patients, whereas for the remaining patients between two to eight fecal samples were collected for the purpose of this study. In total, 97 active and 97 remission samples were available for the analysis. Patient characteristics at time of collection of all 194 samples are given in table 2. Three patients received a course of antibiotics between 1-3 months prior to collection of one of their remission samples (amoxicillin 5 weeks, daptomycin 8 weeks and ciprofloxacin 12 weeks prior to sample collection, respectively), whereas none of the active disease samples were collected within three months after a course of antibiotics.

Microbial composition and diversity

A total of 2,617,664 raw sequences were obtained, and after quality filtering and binning 1,616,532 sequences were retained for further analyses with an average of 8,333 sequences per sample (range 4,938-17,8913 sequences/sample). Sequences were clustered into 6,629 OTUs, subsequently singletons were removed and the data were rarefied to 4,930 sequences/sample to control for variations in sequencing efforts.

The fecal microbiota of remission and active samples did not significantly differ with respect to microbial diversity as assessed by Chao1 (median [interquartile range]: 1077.7 [760.6-1280.0] and 1120.2 [823.2-1307.8, resp.] and Shannon indices (7.0 [6.2-7.5] and 6.9 [6.4-7.7], resp.).

With respect to the microbial composition, both remission and active samples were dominated by the phyla Bacteroidetes (relative abundance 52.9% vs. 49.5%, resp.) and Firmicutes (relative abundance 41.0% vs. 42.9% resp.), followed by Proteobacteria (relative abundance 4.6% vs. 4.0%, resp.) and Actinobacteria (relative abundance 0.7 vs. 0.8%, resp., Supplementary Figure 4.1A). However, the presence of some of the less abundant bacterial phyla differed between the remission and active samples. Fusobacteria could be detected in 31 (32.0%) of the samples collected during active disease, whereas only 6 (6.2%) of the remission samples were positive. In contrast, Verrucomicrobia were more prevalent in remission than in active samples (44.3% vs. 15.5%, resp., Supplementary Figure 4.1B). Also when only examining the baseline samples of the 71 patients (of whom 35 had active disease at baseline), these differences persisted for both the Fusobacteria (37.1% of active vs. 11.1% of remission samples, $p = 0.01$) and Verrucomicrobia (17.1% of active vs. 50.0% of remission samples, $p = 0.003$). The difference in the prevalence of these bacterial phyla was completely driven by the genera *Fusobacterium* and *Akkermansia,* respectively. These genera however did not belong to the dominant microbiota. The microbiota in both remission and active samples was dominated by the genera *Bacteroides, Prevotella* and

Parabacteroides within the Bacteroidetes phylum en members of the Lachnospiraceae and Ruminococcaceae families within the Firmicutes phylum (Supplementary Figure 4.1C).

Random Forest Analysis

We subsequently performed RF analysis to examine whether we could discriminate samples collected during remission and active disease based upon the microbiota composition. First, we reduced the data by including only those OTUs (n = 1,116) that were present in at least 20% of the remission and/or active samples. Subsequently, a first RF analysis was used for the selection of the most discriminatory OTUs between active and remission samples. The RF-analysis assigned a variable importance score to each OTU, indicating to what extend the OTUs contributed to the model. Based on the variable importance profile, fifty OTUs with the highest variable importance scores were selected (figure 4.1).

Table 4.1. Baseline characteristics of CD patients (n=71)

Characteristics	n (%)
Number of samples per subject (%)	
Single sample	14 (19.7)
2 samples	21 (29.6)
3 samples	19 (26.8)
4 samples	8 (11.3)
5-8 samples	9 (12.7)
Male (%)	33 (46.5)
Age (in years; median, range)	44 (18-70)
Disease localization[1] (%)	
L1 (ileal)	23 (32.4)
L2 (colonic)	17 (23.9)
L3 (ileocolonic)	31 (43.7)
Abdominal surgery (%)	
(Partial) colectomy	6 (8.5)
Current smoking (%)	14 (19.7)
Age at diagnosis[1]	
A1 (<16y)	4 (5.6)
A2 (17y-40y)	47 (66.2)
A3 (>40y)	20 (28.2)
Disease phenotype[1]	
B1 (non-stricturing/nonpenetrating)	52 (73.2)
B2 (structuring)	10 (14.1)
B3 (penetrating)	9 (12.7)

[1] according to Montreal classification

Table 2. Medication use, disease location and activity scores for active and remission samples[1] (n=194)

	Remission (n=97)[a]	Active (n=97)[a]
Medication use (%)[b]		
Mesalazine	14 (14.4)	9 (9.3)
Immunosuppressants	39 (40.2)	37 (38.1)
Biologicals	68 (70.1)	44 (45.4)
Antibiotics[b]	3 (3.1)	0 (0.0)
Disease location (%)[c]		
L1 (ileal)	46 (47.4)	23 (23.7)
L2 (colonic)	11 (11.3)	24 (24.7)
L3 (ileocolonic)	40 (41.2)	50 (51.5)
Fecal calprotectin[d]	14.0 (14.0-98.0)	582.0 (259.0-4900.0)
Serum CRP[d]	1.0 (0.0-4.7)	5.4 (0.9-175.0)
Clinical activity index (HBI)[d]	1.0 (0.0-4.0)	3.0 (0.0-15.0)

A. 194 samples were collected from 71 CD patients
B. Used between 1-3 months prior to sampling moment.
C. According to Montreal classification
D. Continuous variables are expressed as median (range).

The performance of the RF classification model based on the most discriminatory OTUs resulted in an area under the ROC curve (AUC) of 0.82 for the validation set, corresponding to a sensitivity of 0.79 and a specificity of 0.73 (figure 4.2). The positive predictive value (PPV) and negative predictive value (NPV) were both 0.76.

The prediction rate of each sample ranges from 0 to 1 and is shown in figure 4.3. Samples with a prediction rate of <0.5 were classified as remission while samples with a classification rate of >0.5 are classified as an active sample. None of the samples had a prediction rate of 0.5. The most discriminant OTUs with their variable importance scores, colored based on their presence in remission or active samples, are depicted in figure 4.1. OTUs belonging to members of *Lachnospiraceae* and *Ruminococcaceae* were found in both remission and active samples. OTUs classified as *Alistipes massiliensis, Faecalibacterium prausnitzii, Bacteroides ovastus* and *Bacteroides uniformis* were associated with remission samples, whereas other OTUs within the genus *Bacteroides*, including *B. fragilis,* were associated with active samples.

The principal component analysis (PCA) on the proximities showed a clear separation between active and remission samples (figure 4.4a). Furthermore, the active samples were found to cluster more tightly together than the remission samples, indicating that the inter-sample variation was smaller in the active as compared to the remission samples. The number of samples of CD patients during remission and active disease that were positive for these discriminative OTUs, as well as the average read numbers of these OTUs per sample, are shown in supplementary

Table S1. Some OTUs show clear differences in mean read numbers (*e.g. Bacteroides ovatus* #4234212 and *Bacteroides* #2949328, while for others the differences are not so distinct (e.g. Lanchospiraceae #2771073). This indicates that the entire set of 50 OTUs contributes to the differentiation between active and inactive CD.

Figure 4.1. 50 most discriminative OTUs, as identified through Random Forest Analysis, to differentiate fecal samples from CD patients during active disease versus remission.

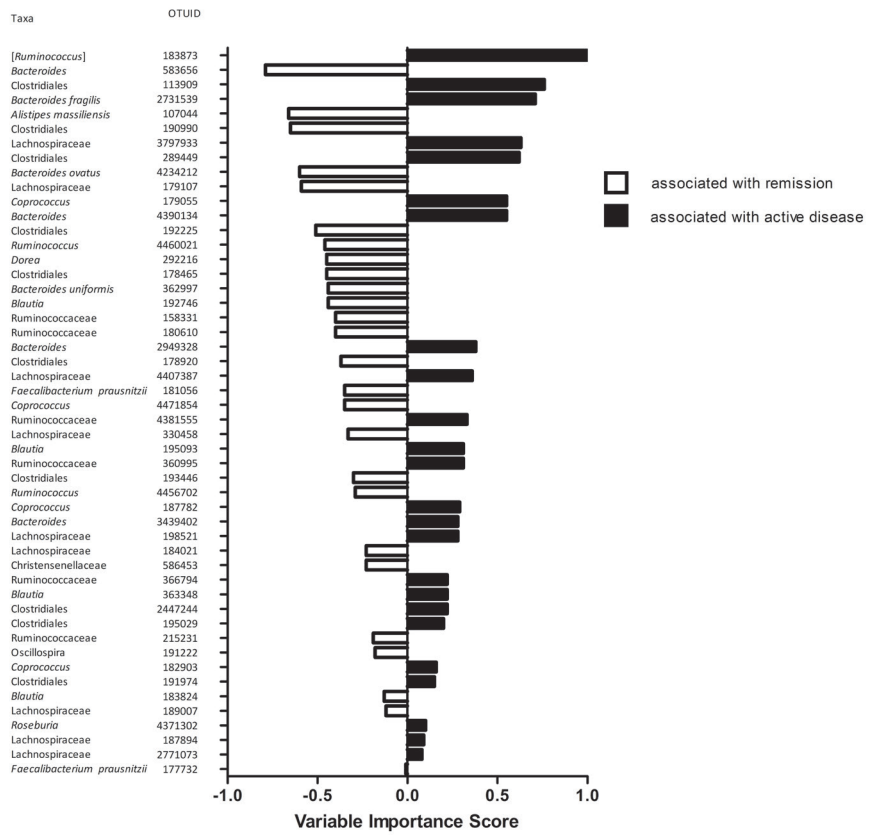
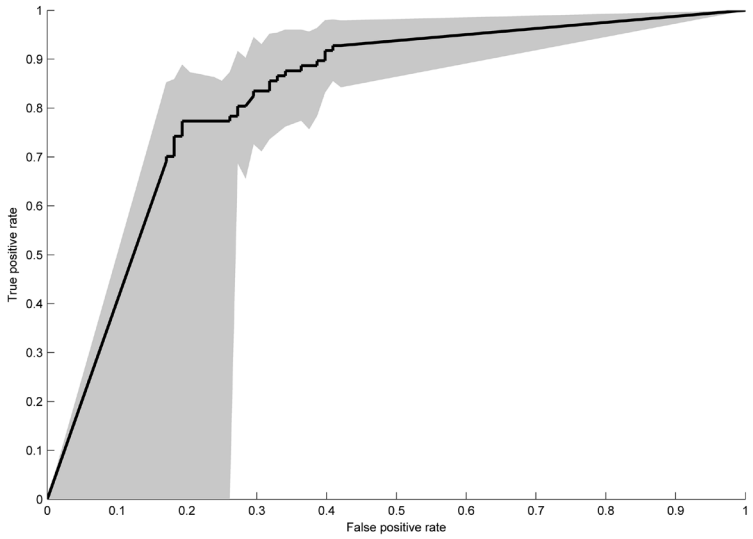
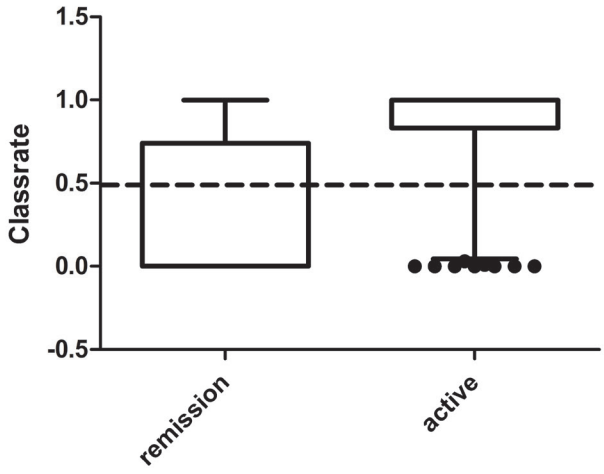


Figure 4.2. ROC curve for the independent validation set (n=88 remission and n=97 active samples) based on the 50 most discriminative OTUs.



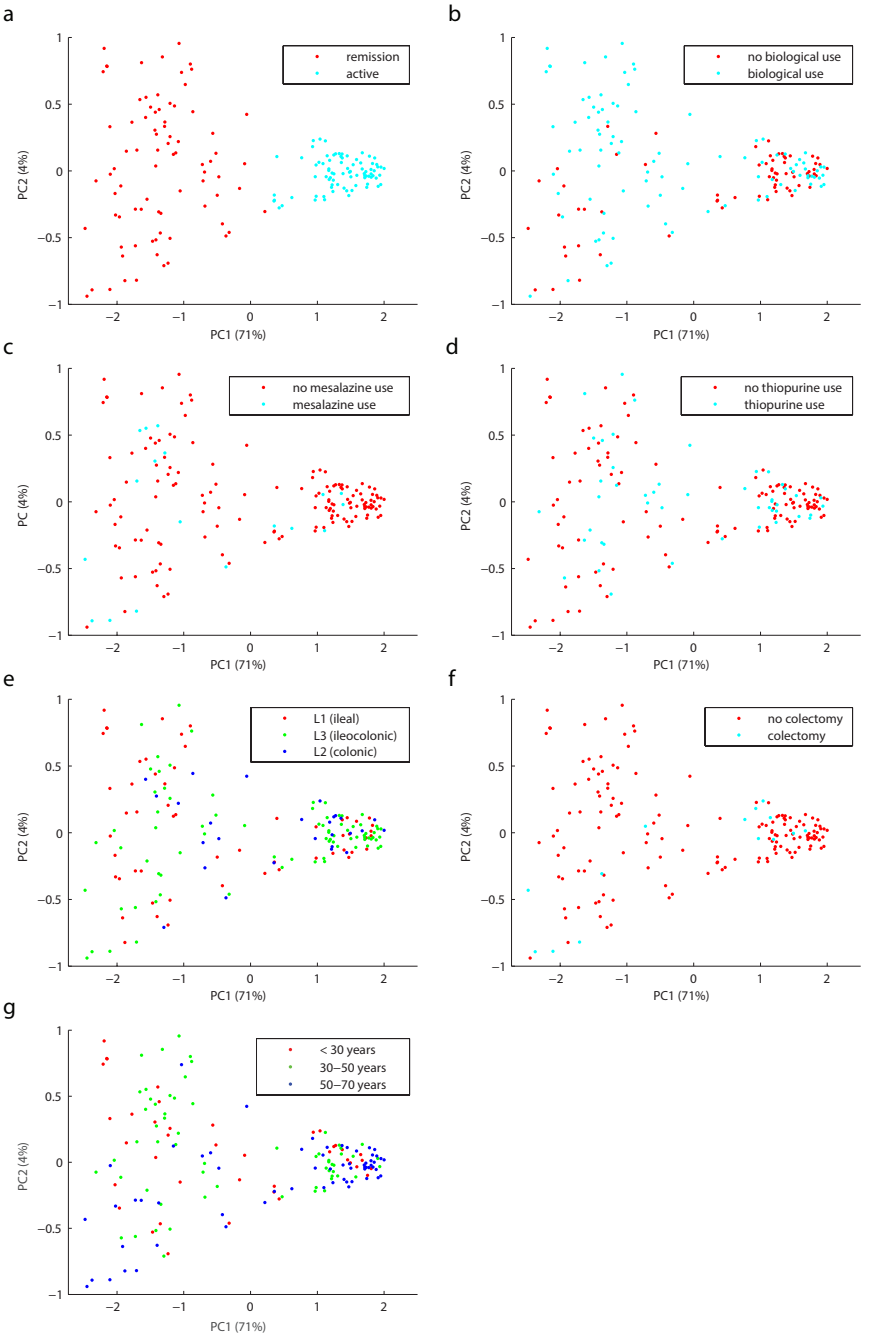
AUC: 0.82, sensitivity: 0.79, specificity: 0.73

Figure 4.3. Classification rate of remission (n=88) and active samples (n=97) from the independent validation set based on the final RF-model.



Classification rates range from 0 to 1. Remission samples with a classification rate < 0.5 were correctly classified as a remission sample. Active samples with an classification rate >0.5 were correctly classified as an active sample.

Figure 4.4. PCA plots based on the proximity matrix from the fecal samples (N=164) of the training set using the 50 most discriminant OTUs.



Samples show a clear separation for active versus remission based on the 50 pre-selected OTUs (a), while no separation was observed for use of biologicals (b), mesalazines (c), thiopurines (d), disease location (e) colectomy (f), and (g) age.

Using the Friedman test, no confounding effect due to medication use (*i.e.* biologicals (p=0.19), mesalazines (p=0.54) and thiopurines (p=0.57)), colectomies (p=0.55), disease location (p=0.98) or age (p=0.45), was observed. The analysis of rMANOVA did not yield significant associations between medication use (biologicals (p=0.52), mesalazines (p=0.55) and thiopurines (p=0.75)), disease localization (p=0.43), colectomies (p=0.72) or age (p=0.72) and the discriminatory set of 50 OTUs. Clustering according to medication use, disease localization, colectomy and age could also not be found in the PCA plots (figure 4.4b-4.4g). CCA analysis showed a strong but non-significant correlation between fecal calprotectin measurements and the 50 most discriminating OTUs (p=0.16, R=0.91).

DISCUSSION

In this study, we demonstrate the potential of fecal microbial profiles as marker for disease activity in patients with CD. Using Random Forest analyses, a combination of 50 bacterial taxa was found to be able to distinguish between active and remission samples with an AUC of 0.82, corresponding to a sensitivity of 0.79 and a specificity of 0.73. Despite the different disease locations and medications used by the study population, the discriminative power of the model was not influenced by these factors, reinforcing that the fecal microbiota has potential as a robust disease activity marker.

A large group of well-characterized CD outpatients from daily clinical practice was included in the present study. Although determination of disease activity by endoscopy is the current standard, this is not feasible in a real-life outpatient follow-up cohort. Therefore, we used a combination of inflammation markers (FC and CRP) and clinical symptoms (HBI) to assess disease activity, which is nowadays well accepted as a surrogate for mucosal inflammation [10,35]. Although previous studies reported an association between specific bacterial taxa and disease severity, others were not able to find such differences [16,17,19–22,24,41]. Most of these studies used univariate analysis methods and compared within and between samples diversity measurements with unsupervised classification methods, which can fail to extract relevant interactions from highly complex data sets. A recent study by Kolho *et al* found a significant correlation between a combination of 9 bacterial taxa and calprotectin concentrations, while no correlation was found with individual bacterial taxa, highlighting the importance of multivariable analysis of microbiota data [42].

Supervised learning techniques, suitable to handle highly complex and sparse data sets, have until recently rarely been used in microbiota data analysis [43]. Random forest uses pattern recognition to discriminate between classes and is able to build predictive models such as needed for biomarker discovery. When applying random forest, we found that a combination of 50 bacterial taxa being able to distinguish active from remission samples in adult CD patients with a sensitivity of 0.79 and specificity of 0.73. The performance of our model was even slightly better than comparable analyses in pediatric IBD patients [28]. Our results support the current notion that a combination of bacterial taxa, rather than specific microorganisms, is involved in CD pathogenesis. Our findings were further supported by the PCA plot showing a clear separation between active and remission samples. Interestingly, the microbiota of active samples was found to be more homogenous than the microbiota of remission samples. This indicates a rather individual microbiota composition in CD patients during remission, while during exacerbation patients have a more common microbiota profile. Although previous studies have demonstrated that IBD specific therapeutic interventions, such as mesalazine, antibiotics and thiopurines, can affect the microbiota [24,44,45], we found no effect of mesalazine, thiopurines

or biological use on the 50 discriminating OTUs as demonstrated by PCA plots. The effect of antibiotics on the 50 discriminating OTUs is unlikely, since none of the patients used antibiotics within a period of 1 month prior to sampling and only three patients, accounting for three fecal samples, used antibiotics between 1-3 months prior the sampling moment. Also no confounding effect of disease location, prior colectomies or age at time of sample collection was found. This supports the potential of this microbial profile as a robust biomarker for active disease. It should however be noted that we cannot exclude any effect of medication use or disease location on the overall microbiota community structure.

The dataset used in our study included multiple measurements for most individuals, which can lead to an overestimation of the results due to the large inter-individual and small intra-individual variation in intestinal microbiota composition [46]. To address this problem, the random forest was performed with a separate training and validation set, in which the model was never trained on part of the samples of one subject while validated on the remaining samples of the same subject [47,48]. However, further validation of our microbial biomarker pattern in an independent cohort, using endoscopy as standard, is needed.

The 50 most discriminatory taxa identified in the present study, include both commensal microorganisms as well as opportunistic pathogens, further indicating that merely detecting presence or absence of specific taxa is not sufficient. The 50 OTUs include several bacterial taxa that have previously been associated with disease activity in CD patients, including Lachnospiraceae, *Ruminococcus*, *Roseburia, Blautia, F. prausnitzii* and *B. fragilis* [18,20,24,28,49]. However, none of the OTUs belonged to the phyla Verrucomicrobia or Fusobacteria, phyla that were shown to differ in abundance between active and remission samples in our study. This can be explained by the low prevalence of the individual OTUs within these phyla, resulting in the exclusion of these OTUs during the data reduction step prior to the RF analysis.

We found *F. prausnitzii* to be associated with remission. Previous studies have demonstrated that a reduction of *F. prausnitzii* is associated with IBD [18,50–57]. Furthermore, multiple studies reported reduced *F. prausnitzii* levels in CD patients during active disease in feces and intestinal tissues, suggesting an association between *F. prausnitzii* and disease activity [18,58,59]. *F. prausnitzii* is known to promote intestinal health by producing butyrate, thus these results suggests an important role of this SCFA in disease activity [60].

In line with other studies, *B. fragilis* was also found to be an important bacterial species to distinguish between patients in remission and patients with active disease [26,61]. Within a longitudinal pilot study, we previously showed a strong increase in the relative abundance of *B. fragilis* in two out of ten CD patients progressing from remission to an exacerbation[24]. Although *B. fragilis* is known as a commensal bacteria with anti-inflammatory properties, a recent

study suggests that enterotoxigenic *B. fragilis* might play a role in active disease by increasing gut permeability [26,62]. Further studies need to be performed to investigate whether enterotoxigenic *B. fragilis* indeed is found more frequently in CD patients during exacerbation.

Papa *et al* applied a RF-based algorithm to discriminate pediatric IBD patients in remission versus those with active disease and found Enterobacteriaceae (associated with disease activity) and Lachnospiraceae, *Ruminococcus, Roseburia* and *Blautia* (associated with remission) to be amongst the most important features to identify disease activity levels in pediatric IBD patients. In line with this study we found members of the *Lachnospiraceae, Ruminococcus, Roseburia* and *Blautia* amongst the most important OTUs, however we could not confirm whether these taxa were associated with remission exclusively. The discrepancies between our study and the study of Papa *et al* could be due to a different population (children versus adults) as well as a different definition of disease activity (PCDAI and PUCAI versus a combination of clinical symptoms and CRP/FCP measurements). Moreover, Papa *et al* collated CD and UC patients together to predict disease activity. Nonetheless, the performances of both models are similar, demonstrating the potential use of the microbiota as a predictive marker.

Since fecal calprotectin is known to correlate well with colonic inflammation, a correlation between fecal calprotectin and the 50 most discriminating OTUs was investigated. We found a very strong correlation between fecal calprotectin and the 50 most discriminating OTUs, which was however not significant. This might be due to a small number of samples. Fecal calprotectin has been reported to correlate well with colonic inflammation, but moderately with inflammation in the proximal colon and small bowel [9,10]. The current study however, thus clearly shows the potential of a bacterial profile consisting of a combination of OTUs as marker for disease activity. As perturbations of the intestinal microbiota are a potential pathophysiological factor in the development of exacerbations, it would be interesting to further investigate the potential of microbial profiling to monitor patients over time.

In conclusion, by applying random forest analysis we found that the fecal microbiota can be used to distinguish adult CD patients based on disease activity. A combination of 50 OTUs was found to be important in the discrimination between samples from remission and active disease, rather than specific bacterial taxa. Establishing a combination of key bacterial taxa unique to disease activity offers the opportunity to use simple and relatively inexpensive methods (*eg.* PCR-arrays) to assess disease activity. Furthermore, using the fecal microbiota as a disease activity marker can lead to new insights in the development of exacerbations and disease pathophysiology. Further studies in which mucosal inflammation is assessed by endoscopy and prospective follow-up studies with IBD patients are warranted to validate our findings.

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SPECIFIC AUTHOR CONTRIBUTIONS

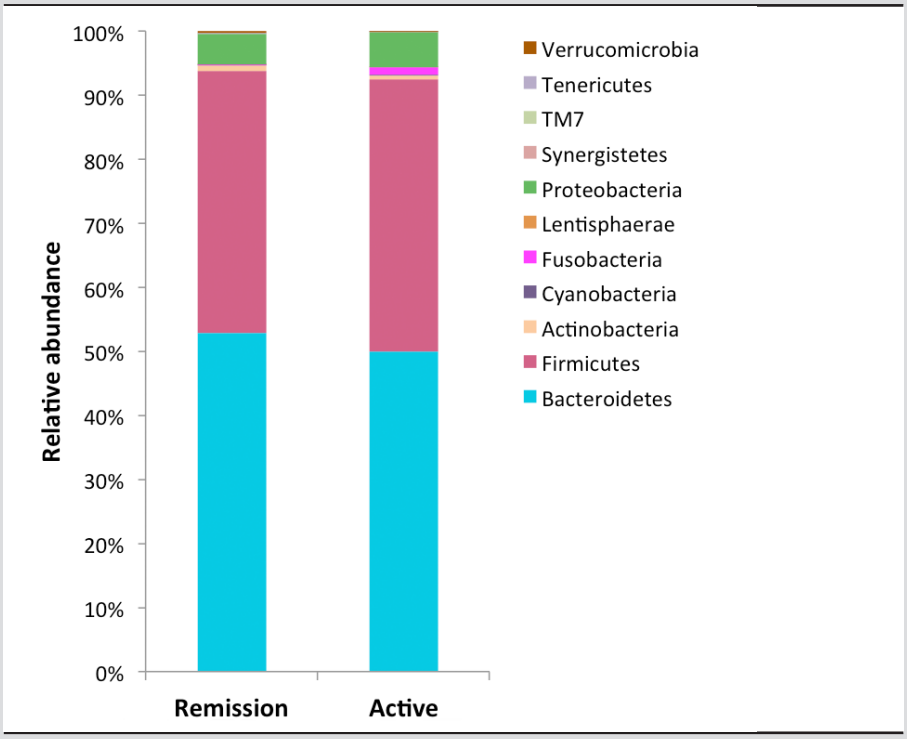
Conceived and designed the experiments: D.I.T., A.S., P.H.S, A.A.M, M.J.P., J.P. and D.M.A.E.J.
Data acquisition: D.I.T.
Analysis and interpretation of data: D.I.T., A.S., J.P. and D.M.A.E.J.
Drafting of manuscript and critical revision of the manuscript for important intellectual content: D.I.T., A.S., P.H.S, A.A.M., F.J.S., M.J.P., J.P. and D.M.A.E.J.
Final approval of the version to be published: D.I.T., A.S., P.H.S., A.A.M., F.J.S., M.J.P., J.P. and D.M.A.E.J.

POTENTIAL COMPETING INTEREST

A. Masclee receives grants from DSM, Grunenthal, Abbott and Danone. M. Pierik acted as a consultant for Takeda in the past, is a former lecturer for Abbvie, Falk, MSD and Ferring. The other authors declare no conflict of interest.

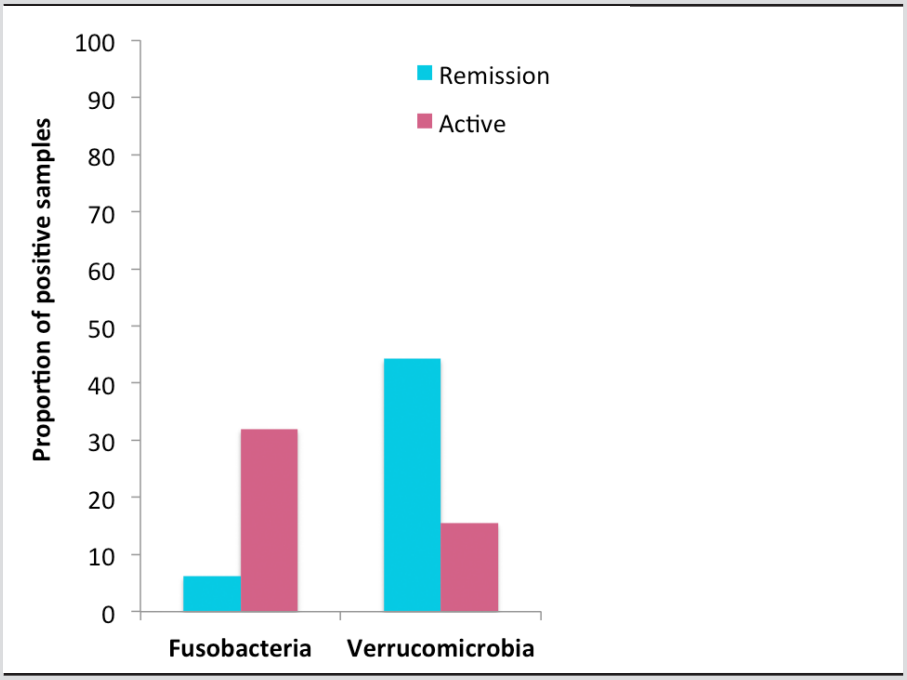
SUPPLEMENTAL INFORMATION

Supplementary Figure 4.1A. Microbial composition of samples from Crohn's Disease patients during remission and active disease.



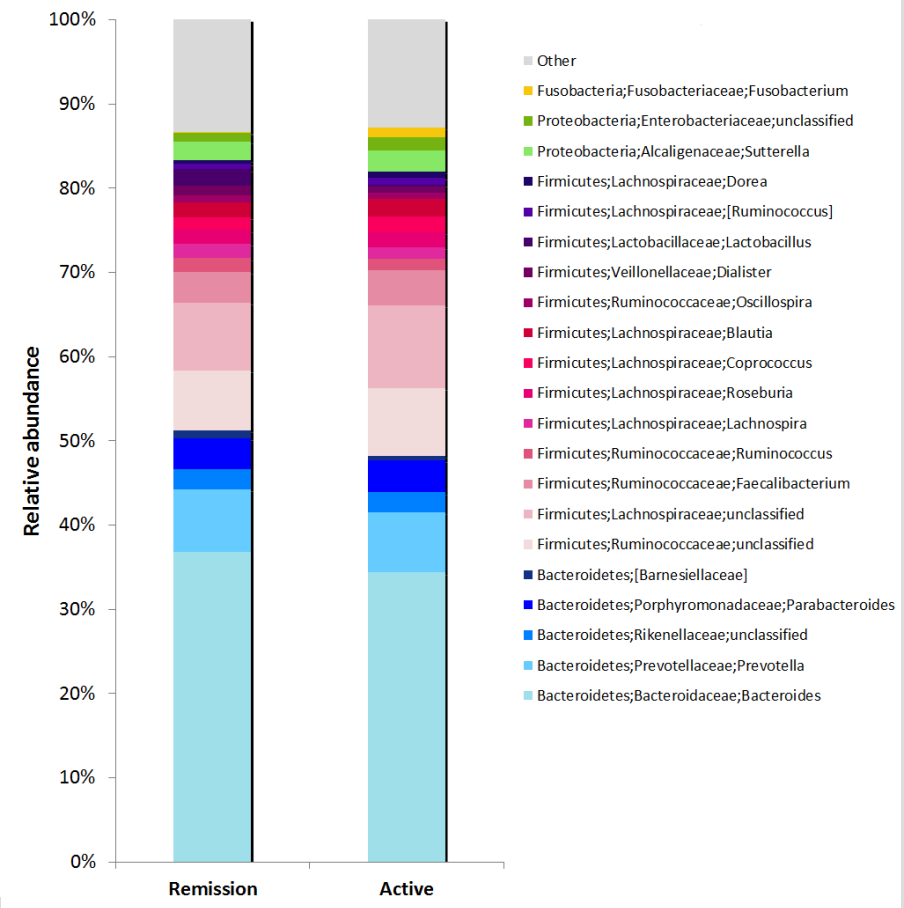
Relative abundance of bacterial phyla.

Supplementary Figure 4.1B. Continued.



Proportion of samples containing Fusobacteria and Verrucomicrobia.

Supplementary Figure 4.1C. Continued.



Relative abundance of the most dominant bacterial genera (colored according to the respective phyla Bacteroidetes (blue), Firmicutes (pink - red – purple), Proteobacteria (green), Fusobacteria (yellow).

Table S1. Proportion of samples positive and mean (std) read numbers in positive samples for each of the discriminatory set of 50 OTUs for individuals in active and remission.

OTUs numbers	Active CD samples (N=97)		Remission CD samples (N=97)	
	Number of samples positive for OTU	Mean read number (STD)*	Number of samples positive for OTU	Mean read number (STD)*
2949328	54	11,06 (22,5341808854039)	30	12,400 (16,1001177978128)
3439402	30	15,17 (35,1990236280471)	22	13,864 (31,4904300724393)
192225	23	2,04 (1,63702226534437)	9	1,556 (0,52704627669473)
289449	31	4,81 (5,21164948193762)	9	2,111 (1,05409255338946)
4381555	22	5,64 (8,79147560367674)	8	11,375 (13,8763673714907)
292216	21	1,43 (0,870139561876632)	10	1,100 (0,316227766016838)
2447244	38	5,63 (9,09832492047101)	22	5,045 (5,35836926798828)
586453	5	1,40 (0,547722557505166)	22	1,364 (0,902137912878521)
193446	22	1,55 (0,962500351380158)	20	1,350 (0,812727700887249)
2771073	27	1,30 (0,541707756495413)	14	2,571 (2,40877516638598)
363348	24	1,71 (1,73152776639507)	10	1,100 (0,316227766016838)
4234212	13	2,31 (1,9315199276138)	38	7,974 (15,6265312194241)
215231	8	1,13 (0,353553390593274)	25	1,560 (0,820568908339411)
178920	19	1,26 (0,452413928358864)	27	1,407 (0,747264717757073)
583656	25	3,92 (3,49904748943671)	2	1,000 (0)
180610	25	1,28 (0,541602560309064)	12	1,167 (0,577350269189626)
330458	27	1,89 (2,00640001635791)	24	1,625 (1,17260393995586)
179055	23	2,13 (1,57550418556574)	11	1,273 (0,467099366496914)
4371302	27	1,52 (1,12216721537356)	22	1,864 (1,90976075653108)
177732	9	2,33 (1,5)	19	1,368 (0,683985568056769)
362997	6	6,33 (9,81155781039212)	23	4,478 (4,27327386967152)
4460021	6	1,83 (1,32916013582513)	33	2,545 (2,69363728407925)
183824	24	1,42 (0,775531608229038)	18	1,278 (0,460888598962477)
195093	32	1,63 (1,07012209131602)	14	1,500 (0,85485041426511)
184021	28	2,07 (1,84448269227889)	24	1,958 (1,68055805086252)
187782	21	1,33 (0,658280588604383)	10	2,000 (2)
182903	27	1,81 (1,07549763211973)	17	1,765 (1,25146972419961)
4407387	22	1,68 (0,994574023980865)	9	1,111 (0,333333333333333)
178465	19	1,95 (1,61498827689334)	24	2,458 (2,37704907792005)
4471854	43	1,77 (1,26936822122784)	40	1,725 (1,26059611458332)
107044	30	8,80 (20,0935742001748)	19	37,000 (56,6048682633492)
158331	11	2,91 (2,07145096270925)	32	3,844 (3,20392274684362)
191974	35	1,66 (1,34913604474378)	17	1,529 (0,874474632195206)
179107	11	1,27 (0,646669790682863)	19	1,211 (0,535303379031311)
113909	49	2,94 (3,38752714174432)	33	2,788 (2,63103562971855)
4390134	33	6,76 (10,5030659304507)	22	9,227 (19,6757811442485)
360995	23	1,30 (0,558795995356872)	16	1,063 (0,25)
192746	38	4,47 (5,17131820773871)	28	1,679 (1,46700933947825)
3797933	26	1,15 (0,464095480892257)	9	1,333 (0,707106781186548)
195029	27	2,93 (5,2909641838045)	12	2,333 (1,77525072919719)
189007	27	1,30 (0,541707756495413)	19	1,158 (0,374634324632678)
187894	28	2,43 (2,20149059651883)	12	1,167 (0,389249472080762)
190990	74	10,36 (15,5559743395495)	52	9,058 (9,27449266418271)
181056	32	3,38 (2,73271695121977)	35	2,943 (4,09406210722494)
191222	11	1,27 (0,646669790682863)	30	2,200 (1,93693679244601)
198521	27	1,96 (1,34397463006852)	22	1,591 (0,908116360757433)
366794	30	2,67 (3,42740812229615)	21	4,143 (5,91849401694143)
4456702	4	2,25 (2,5)	23	3,130 (4,71269415154516)
183873	21	1,33 (0,730296743340222)	7	1,571 (0,786795792469443)
2731539	22	3,36 (5,12273601139316)	7	46,429 (75,3720928524104)

*Mean (STD) read numbers are based upon samples positive for the specific OTU only.

5

Strong correlation between the fecal microbiota and volatile metabolites in breath of Crohn's disease patients

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ABSTRACT

Background: Recent developments in microbial sequencing technologies have instigated the research on the human gut microbiome in health and a variety of diseases. In addition to the microbiota composition, also its metabolic capacity is very important for host health by the production of *e.g.* volatile metabolites such as short chain fatty acids. Such metabolites are partly excreted in the faeces, but can also be absorbed and excreted in exhaled air. Although profiles of both the gut microbiota and breath volatiles have been associated with several diseases, a direct link between them has not yet been investigated.

Methods: Samples were available from previous studies in patients with Crohn's disease (CD), which found discriminating profiles for exhaled volatiles and the faecal microbiota in active versus quiescent CD. From 68 CD patients repeated faecal and breath samples were collected at various stages of the disease, resulting in 92 active and 92 remission samples. The microbiota composition was assessed by pyrosequencing of the 16S rRNA V1-V3 gene region. Volatile metabolites in breath were detected by gas chromatography-time-of-flight mass spectrometry. Canonical Correlation analysis (CCA) was used to assess the relation between exhaled volatile metabolites and bacterial species in faecal samples of CD patients during active and inactive stages of the disease.

Results: CCA analysis identified 18 volatiles in breath and 19 faecal bacterial taxa, which showed a strong and significant correlation in samples from CD patients during active disease ($R=0.91$ with p-value $3.5*10^{-4}$). In samples obtained during inactive CD, 17 volatiles and 17 bacterial taxa had the highest contribution to the overall correlation ($R=0.96$ with p-value $2.8*10^{-4}$). Among these sets, only 9 volatile metabolites and 3 faecal bacteria taxa were identified in both active and inactive CD.

Conclusion: This is the first study that shows a strong relation between profiles of the intestinal microbiota and volatile metabolites in exhaled breath, which were found to differ between active and quiescent CD. Unravelling this link is essential to increase our understanding on the functional effects of the microbiome and may provide new leads for microbiome-targeted intervention studies and/or disease monitoring.

INTRODUCTION

The human gut hosts a complex ecosystem, which contributes to human health and disease [1, 2], for example by colonization resistance, its impact on the maturation and functioning of the intestinal epithelium and immune system and by its large metabolic capacity. This involves complex microbe-microbe and microbe-host interactions, leading to a variety of metabolites such as secondary bile acids, choline and saccharolytic and proteolytic fermentation products that can affect host health [3, 4]. This can be further influenced by environmental factors such as dietary intake and drug use [5, 6]. Unravelling the link between the gut microbiome and host metabolism is essential to increase our understanding on the functional effects of the microbiome in and beyond the gastrointestinal tract and could provide new leads for microbiome-targeted intervention studies [7-10].

Metabolomic analyses applied to different biological samples (*e.g*. faeces, urine or blood) enable the identification and characterization of metabolites produced by intestinal microbes and provides insight in microbial activity. Volatile Organic Compounds (VOCs) belong to carbon based metabolites [11] and are produced during normal and deviant (for instance inflammatory) metabolic processes occurring in the human host [12, 13], but can also results from exogenous compounds (*e.g*. uptake of environmental contaminants) and microbial metabolism. Upon formation in the body, volatile metabolites reach the blood-lung barrier through the blood stream and are quickly excreted via the airways. Many VOCs in exhaled air, including hydrocarbons and aldehydes, are also produced by the intestinal microbiota [14]. Numerous food ingredients present in the large intestine, especially non-digestible oligo- and poly- saccharides, which are fermented by the intestinal microbiota, lead to the production of various volatile metabolites including short chain fatty acids (SCFAs) such as acetate, butyrate and propionate, but also alcohols such as ethanol and propanol [5, 15, 16]. The concentration and products of bacterial fermentation, which can be detected in exhaled breath [17], can differ between health and disease or between disease courses. Thereby, the resulting volatile metabolites, which may reflect the microbial metabolic activity in addition to host processes, can provide further insight into the microbial activity in health and disease [18].

Previously, we found specific discriminating VOC as well as faecal microbial profiles for Crohn's disease (CD) patients with active versus quiescent disease [19, 20]. This chronic relapsing inflammatory disorder is hypothesized to result from the interplay between the microbiota, immune function, host genetics and environmental factors [21]. The microbiota is likely to play a role in the development or progression of active disease. We hypothesize that there are several volatile metabolites in breath that are directly or indirectly related to the microbiota composition and that the presence and abundance of such metabolites in exhaled air may differ between active and inactive CD disease stage. In this proof-of-principle paper we present for

the first time the findings of an integrated experimental and bioinformatics technique to find the relation between the faecal microbial compositions and exhaled volatile metabolites in CD patients in active and inactive stage of the disease. Unravelling this link is essential to increase our understanding on the functional effects of the microbiome and may provide new leads for disease monitoring.

MATERIAL AND METHODS

Study population

Samples of 68 CD patients participating in a 1-year follow-up study as part of a prospective cohort of IBD outpatients of the population-based IBDSL cohort [20, 22], were available for analyses in the current study. The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center (the Netherlands; NL24572.018.08) and all patients gave written informed consent prior to participation. All patients had an established diagnosis of CD based on clinical, endoscopic, histological and/or radiological criteria [23]. Active disease was defined by a Faecal Calprotectin (FC)≥250 µg/g [24]. Remission was defined by a Harvey-Bradshaw Index ≤4 in combination with both serum C-reactive protein (CRP) <5 mg/l and FC<100 µg/g. Patients were assessed at study entry and subsequently at regular (3 monthly) visits or when a flare occurred. At each visit clinical data, blood, faecal and breath samples were collected. CRP and FC were analyzed routinely by the laboratory of Clinical Chemistry. Faeces was collected by the patients at home and brought to lab clinic within 12 hours after defaecation. Upon arrival, part of the sample was sent to the laboratory of Clinical Chemistry for routine analysis of FC. The remaining part was aliquoted and frozen directly at -80°C for microbiota analyses using 454 sequencing.

DNA isolation of faecal samples

Approximately 200 mg of frozen aliquots of faecal samples was added to vials containing PSP lysis buffer (Stratec Molecular, Berlin, Germany), 0.5 g of 0.1 mm zirconia/silica beads and 4 glass beads of 3.0-3.5 mm (BioSpec, Bartlesville, USA). The homogenization of the faecal samples was performed in a MagNALyser instrument (Roche, Basel, Switzerland) in three cycles of 1 min at a speed of 5500 rpm. Between each cycle the samples were kept on ice for one minute. DNA isolation was continued using the PSP Spin Stool Kit (Stratec Molecular, Berlin, Germany). Finally, DNA was eluted in 200 µl TE-buffer.

454 pyrosequencing

Amplification of the V1-V3 16S rRNA amplicons was implemented using forward primers containing of a 9:1 ratio mixture of 8F and 8F-Bif, respectively, and reverse primer 534R as described previously.[25] The PCR reaction was done using 1x FastStart High Fidelity Reaction Buffer, 1mM dNTP solution, 1.8 mM $MgCl_2$, 5 U FastStart High Fidelity Blend Polymerase (Roche, Indianapolis, USA), 0.2 µM forward primer, 0.2 µM reverse primer and 1 µl of template DNA using the following settings: 3 minutes of denaturation at 94°C followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 45 seconds and extension at 72°C for 5 minutes. The final step consisted of elongation at 72°C for 10 minutes. The purification of the amplicons was performed using AMPure XP purification according to the manufacturer's instructions and eluted in 25 µl TE. Amplicon concentrations were obtained by Quant-IT Pico Green dsDNA reagent kit

(Invitrogen, New York, USA) via the Victor3 Multilabel Counter (Perkin Elmer, Waltham, USA). Subsequently, amplicons were mixed in equimolar concentrations to establish an identical representation of each sample for the emulsion PCR (emPCR). After emPCR (Titanium emPCR Kit (Lib-L)), pyrosequencing was executed according to the manufacturer's instructions (Roche, Brandford, USA).

Sampling and analysis of exhaled air

Exhaled air was collected using 5L bags (Tedlar Bag, SKC Ltd, Dorset, UK). One hour before sampling, eating, smoking and exercise were not allowed. All patient samples were collected at the same location at random time in order to prevent the appearance of a background bias. Within 1 hour after collection the volatiles from bag were trapped onto carbon-filled stainless steel sorption tubes (Markes International, Llantrisant Business Park, UK). The air-tight capped tubes were stored at room temperature until further analysis. Volatile metabolites were measured by thermal desorption-gas chromatography combined with *time-of-flight* mass spectrometry (GC-*tof*-MS)[26]. The experimental settings for GC and tof-MS can be found elsewhere.[11] The compounds after separation by GC were identified by *tof*-MS (Thermo Electron Tempus Plus time-of-flight mass spectrometer, Thermo Electron Corporation, Waltham, USA).

Data handling

In case of 16S rDNA data, the first step consisted of reducing the error rates by filtering the raw pyrosequencing data via Mothur version 1.32.1 [27]. For further analysis only sequences with perfect proximal primer fidelity, a minimum average quality score of 25 over a window size of 50 nucleotides, a read length between 200 and 590, a maximum of one ambiguous base call and a maximum homopolymer length of 6, were included. UCLUST algorithm was used to cluster sequences into operational taxonomic units (OTUs) based on 97% similarity against the Greengenes reference set version August 2013 in Qiime 1.8 [28]. De novo OTUs were suppressed (*i.e.* sequences were discarded if they did not cluster with any of the reference sequences). The final outcome led to a data matrix containing sample/patients in rows and OTUs in columns.

The raw chromatograms obtained by GC-*tof*-MS (called breathograms) were preprocessed to moderate the effect of various artifacts such as noise and baseline, column bleeding, and chromatographic drift. The detailed description of the preprocessing steps can be found elsewhere [29]. Shortly, parts of the chromatograms were excluded due to noisy mass spectra at the beginning of the chromatograms (retention index <1.3 minute) and column bleeding at the end of each chromatogram (retention index in minutes >23). The next steps consisted of noise removal and baseline correction (removing chromatographic background) using wavelets and P-splines, respectively [30, 31]. For each peak in Total ion current (TIC) chromatogram the area under the peak is calculated. These areas calculated for each peak are matched from sample to sample based on the similarity in retention time and Pearson correlation between

the mass spectra. A high correlation (superior to 85%) was used to associate the peaks as the same compounds. The last step allowed representing chromatographic data as data matrix with samples/patients in rows and relative concentration of measured volatiles metabolite (ion count) in columns.

Statistical analysis via Canonical Correlation Analysis

In order to assess the relationship between volatiles in exhaled breath and the faecal microbiota, Canonical Correlation Analysis (CCA)[32, 33] combined with sparse Principal Component Analysis (PCA) was used [34, 35]. First, PCA was implemented as dimension reduction technique, followed by CCA analysis.

The CCA technique is able to find the relationship between two multivariate sets of variables measured for the same set of samples, and can be considered as the extension of bivariate correlations. The main aim of CCA is to find two new bases (new directions) for each data set, which are a linear combination of the original parameters. CCA finds the two new bases (called canonical variate) in which the correlation between the original parameters in two datasets (*i.e.* exhaled volatile metabolites and faecal microbiota), is maximized. The output can be presented in a CCA score plot using the canonical variates obtained for volatile molecules in breath and faecal microbiota using only the statistically significant canonical variates. In CCA score plot each point corresponds to the combined information from breath and faecal sample collected at a single time-point from the same patients. The shape of the cloud indicates the correlation between two blocks of data (here volatile molecules in breath and faecal microbiota). The more the cloud of points is structured around a straight line, the more relevant the relation between the two blocks of data is. The contribution of the original parameters to the correlation structure between two blocks of data can be represented via canonical coefficients.

The volatile metabolites and faecal bacteria contributing the most to the correlation between two sets of data were selected by sensitivity analysis based on backward elimination of the parameters. The correlation and its significance were evaluated after each removal of the least influential parameters from exhaled volatile metabolites and faecal microbiota data. The procedure of eliminating the parameters was terminated if correlation and its significance were considerably lost [36].

RESULTS

Study population

A total of 184 faecal and breath (92 active and 92 remission) samples of 68 CD patients (18-70 years) were included in this study. Demographic and phenotypic characteristics of the 68 CD patients at time of inclusion are shown in table 5.1.

Sequencing data

Raw faecal microbiome data contained a total of 2,617,664 sequences. Upon quality filtering and binning of raw data, a total of 1,616,532 sequences with an average of 8,284 sequences per sample (range 4,938 - 17,893 sequences/sample) remained for downstream analysis. The sequences were clustered into 6,629 OTUs. The faecal microbiota of active and remission samples were dominated by Bacteroidetes (relative abundance 49.7% vs. 52.5%, resp.) and Firmicutes (relative abundance 42.9% vs. 41.3%, resp.), followed by Proteobacteria (relative abundance 5.2% vs. 4.8%, resp.) and Actinobacteria (relative abundance 0.6% vs. 0.8%, respectively).

Breath analyses

The 184 exhaled air samples contained 650 compounds. The majority of all available VOCs was only detected in a limited number of samples. Therefore, a compound was only kept for further analysis if it could be detected in at least 20% of samples in one of the experimental groups or time points [10], resulting in a final data matrix containing 259 VOCs.

Canonical Correlation Analysis of breath and microbiome data

The CCA between faecal microbiota and volatile metabolites in breath was performed separately for samples obtained during active and remission stage of the disease. First, the CCA analysis was done between all exhaled volatiles and OTUs. The combined analysis of sparse PCA and CCA analysis showed a statistically significant correlation of 0.85 (p-value 0.0015) and 0.82 (p-value 0.001) for inactive and active CD samples, respectively (supplemental figure S5.1A-B).

As the relative abundance was very low for the majority of OTUs (clustered at 97% sequence similarity), we subsequently combined OTUs based on their taxonomy. Moreover, including in the multivariate analysis several OTUs that are assigned (at 97% sequence similarity) to the same species creates a lot of redundancy and increases the collinearity of the data. Therefore, OTUs belonging to the same species were joined into species-level OTUs, while OTUs that lacked species-level taxonomy were combined into genus-level OTUs. This will further be referred to as faecal bacterial taxa. OTUs that could not taxonomically be classified down to the genus-level were excluded. The classification into taxa is more robust to sequencing error and reduces the dimensionality of microbiome data significantly, finally resulting in 166 different faecal bacte-

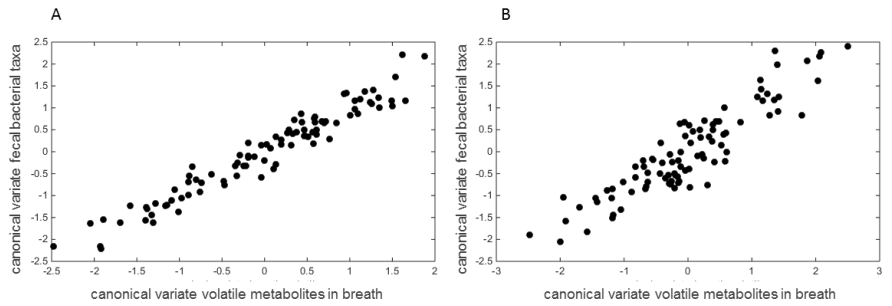
rial taxa. These consisted of 94 genus-level OTUs and 72 species-level OTUs. Subsequently, a second CCA analysis (combined with sparse PCA) was performed on these faecal taxa and the 259 volatile metabolites in breath. The analysis revealed significant correlation of 0.9 (p-value 0.00012) and 0.89 (p-value 0.001) for quiescent and active disease, respectively. Sets of 18 and 17 volatile metabolites in breath and 19 and 17 faecal bacterial taxa were designated as having the highest contribution to the overall correlation for samples of CD patients obtained during active and inactive disease, respectively. The sets volatile compounds and faecal bacterial taxa contribution were selected based on sensitivity analysis [36]. A third CCA analysis was then performed, including only these most important volatiles and faecal bacteria (see also score plots in figures 5.1A-B). In these plots, the x-axis corresponds to canonical variate for volatile metabolites in exhaled breath, while the y-axis to canonical variate for faecal microbiome data. Each point in the figures corresponds to the combined information from breath and faecal sample collected at a single time-point from the same CD patients. The CCA analysis showed a significant correlation between volatile metabolites in breath and faecal bacterial taxa of R=0.91 with p-value of $3.5*10^{-4}$ and R=0.96 with p-value of $2.8*10^{-4}$ for active and inactive CD patients, respectively for the first canonical correlation coefficient.

Table 5.1. Baseline characteristics of CD patients (n=68)

Description	Number of subjects
Male (%)	30 (44)
Age (in years; median, range)	48 (18-70)
Disease localisation[1] (%)	
L1 (ileal)	22 (32)
L2 (colonic)	16 (2)
L3 (ileocolonic)	30 (44)
Abdominal surgery (%)	
(partial) colectomy	6 (9)
Current smoking (%)	14 (20)
Age at diagnosis[1]	
A1 <16y	4 (6)
A2 17y-40y	45 (66)
A3 >40y	19 (28)
Disease phenotype[1]	
B1 non-stricturing/nonpenetrating	50 (74)
B2 stricturing	9 (13)
B3 penetrating	9 (13)

[1] According to Montreal classification, defined at time of sampling. The values are presented as median and range for continuous variables and numbers and percentages for categorical variables.

Figure 5.1. The canonical correlation score plot using the first canonical variates of selected sets of the most important volatiles in breath (x-axis) and faecal bacterial taxa (y-axis) for: A) inactive CD patients; B) CD in active stage.

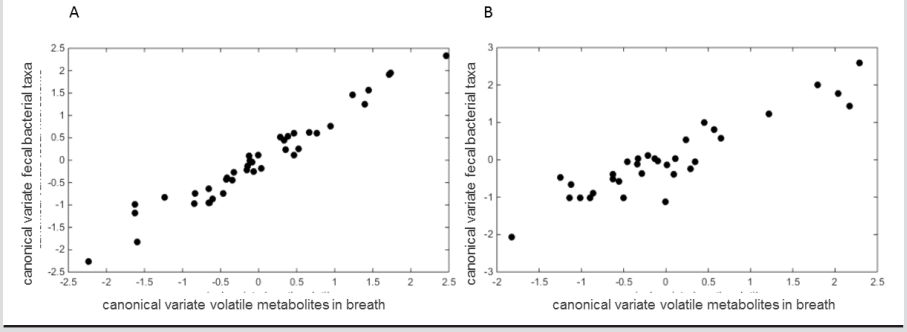


Each point in the figure represents the combined information of a single breath and faecal sample collected at a single follow-up moment from the same CD patient. In both plots there is clear structure in the presented data. The CCA analysis revealed significant correlation between volatiles in exhaled breath and faecal bacterial taxa with R=0.96 and p-value $2.8*10^{-4}$ for CD in remission and R=.0.91 with p-value $3.5*10^{-4}$ for active CD. The first canonical correlation coefficient explains 92% of variance for patients in remission and 83% of variance for active CD. The CCA analysis was performed for the set of 18 and 17 breath volatiles, and 19 and 17 faecal bacterial taxa for active CD and remission, respectively.

Although, in both disease stages the relation between volatiles in breath and faecal microbiome is strong, the results obtained for active CD revealed more overall variations as indicated by the larger spread of the data points (figure 5.1B).

As the CD patients were sampled several times over one year, this may have affected the correlations shown in figures 5.1A-B. Therefore, the CCA analysis was repeated based on only one measurement per patient using the same sets of 17 and 18 breath volatiles, and 17 and 19 faecal bacteria taxa for CD in remission and active disease, respectively. The single sample per patient that was included in CCA analysis was selected randomly and the final outcomes represent the average of 500 CCA iterations. The resulting CCA score plots are shown in figures 5.2A-B for active and inactive CD, respectively. Interestingly, the correlation between faecal bacteria and volatile metabolites in breath is preserved. (R=0.93 and p-value $1.5*10^{-4}$ for CD in remission and R=.0.91 with p-value $2.1*10^{-4}$ for active CD).

Figure 5.2. The canonical correlation score plot, based on only one measurement per patient, for the first canonical variate of selected set of 17 (for panel A) and 18 (for panel B) volatiles in breath (x-axis) and selected sets of 17 (for panel A) and 19 (for panel B) faecal bacterial taxa (y-axis) of faecal bacteria: A) inactive CD patients; B) CD in active stage.



Each point in the figures represents breath and faecal sample of CD patients. In both plots there is clear structure in the presented data. The CCA analysis revealed significant correlations between volatiles in exhaled breath and faecal bacteria taxa with R=0.93 and p-value $1.5*10^{-4}$ for CD in remission and R=.0.91 with p-value $2.1*10^{-4}$ for active CD. The first canonical correlation coefficient explains 86% for patients in remission and 82% of variance for active CD.

The chemical identification and taxonomic assignment of the volatiles in breath and faecal bacteria that contributed most to the overall relation are shown in figure 5.3A and 5.4A. The exhaled volatiles and faecal bacterial taxa that were most contributing to the correlation in active and remission CD samples are only partly overlapping (panel A in figures 5.3 and 5.4, indicated in bold). We found 9 volatile metabolites and only 3 faecal bacterial taxa to be overlapping between active and inactive disease. This outcome indicates that the relation between volatiles in breath and faecal bacteria is in large part dependent on the disease stage (*i.e.* active versus quiescent disease).

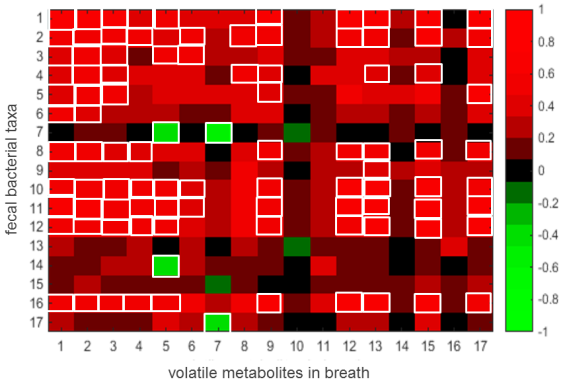
In order to show the inter-omics relation, a spearman pair-wise correlation was calculated between the most contributing sets of 17 and 18 breath metabolites and 17 and 19 faecal bacterial taxa in inactive and active disease, respectively. The correlations were first represented as heatmaps and next as networks. The heatmaps showing the correlations between individual bacterial taxa and volatile metabolites in breath are shown in figure 5.3B and figure 5.4B for CD in remission and active, respectively. The significant correlations after a Benjamini–Hochberg post-hoc correction for multiple testing are indicated as white frames. Interestingly, the vast majority of the individual correlations between faecal bacteria and volatile metabolites in breath were positive.

Figure 5.3.

A

Nr.	Volatile metabolites	Nr.	Fecal bacterial taxa
1	**Ethanol**	1	***Bacteroides uniformis***
2	**Pentane**	2	*Parabacteroides distasonis*
3	Unknown	3	***Prevotella copri***
4	Ethanol+unknown	4	*Bifidobacterium longum*
5	Unknown	5	*Bacteroides barnesiae*
6	**Isoprene**	6	*Veillonella parvula*
7	Methylcyklohexane	7	*Alistipes indistinctus*
8	**Phenol**	8	Unclassified *Odoribacter*
9	D-limonene	9	Unclassified *Lactobacillus*
10	**Pentanoic acid**	10	**Unclassified *Blautia***
11	**Propanoic acid**	11	Unclassified *Coprococcus.*
12	**Octane**	12	Unclassified *Dorea*
13	**Acetic acid**	13	Unclassified *Pseudobutyrivibrio*
14	n-propylcyclohexane	14	Unclassified rc4-4
15	Decane	15	Unclassified *Anaerotruncus*
16	Terpen	16	Unclassified *Oscillospira*
17	**Tridecane**	17	Unclassified *Bilophila*

B



A) The chemical identification of the 17 breath metabolites and taxonomy of 17 faecal bacterial taxa that have the highest contribution to the correlation between volatile compounds in breath and faecal microbiome for inactive CD. Breath volatiles and faecal taxa that were identified as being among the most contributing to the correlation in both active and inactive CD samples are highlighted in bold. B) The Spearman correlation heatmap between 17 selected sets of breath metabolites and 17 bacterial taxa for inactive CD. The numbers in the heatmap correspond to names of the selected breath metabolites and faecal bacterial taxa in panel A. The colours in the heat maps correspond to the positive correlation for red and negative correlation for green. The white squares designate the significant correlation (p-value<0.05) between breath metabolites and faecal bacteria after Benjamini–Hochberg post-hoc correction.

Correlation networks were performed between the same selected sets of breath metabolites and faecal bacterial taxa in inactive and active disease using Cytoscape version 3.4[37]. The correlation networks are shown in figures 5.5 A-B for inactive and active CD respectively. In both networks, a complex relation between the faecal bacterial taxa and exhaled volatiles can be observed. Using a threshold of Spearman correlation r >=0.2, we generated a list of 190 and 168

unique inter-omics Spearman correlations from inactive and active CD data, correspondingly. Note that the correlations between volatiles in breath to volatiles in breath or faecal bacteria to faecal bacteria are not represented here.

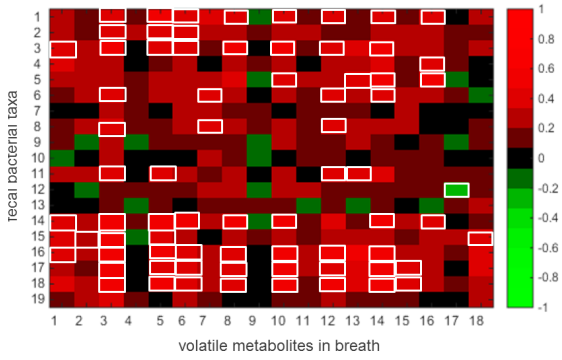
In the active CD samples for example, the level of acetic acid in breath correlated to the abundance in *Blautia spp.*, while decadiene was clearly linked to *Bacteroides* spp. *Clostridium citroniae* is negatively correlated to a branched alkane (C_9C_{20}) whereas other unclassified *Clostridium* were related to the level of acetic acid, 1-pentanol and n-heptane. In remission samples, we also observed *Blautia* being highly correlated to the level of acetic acid, as well as to pentane. Pentane was also highly correlated to the abundance of *Parabacteroides distasonis*. Ethanol is known to be related to a number of microorganisms. This is confirmed here with relevant correlations between ethanol and *Bacteroides uniformis*, *Prevotella capri, Parabacteroides distasonis*, *Bifidobacterium adolescentis* and unidentified members of *Oscillospora*, *Coprococcus Blauta*, *Bilophilia*, *Ofdoribacter* and *Lactobacillus*. Interestingly isoprene seems to be independent of the microbiome, associated with an active disease, whereas it is part of a complex network in remission, where isoprene is correlated to 12 of the selected OTUs, including *e.g*. *Bacteroides uniformis* or *Prevotella copri*. Three bacterial taxa, *Alistipes indistinctus*, *Bilophilia* and *rc44* showed a negative correlation to methylcylclohexane.

Figure 5.4:

A

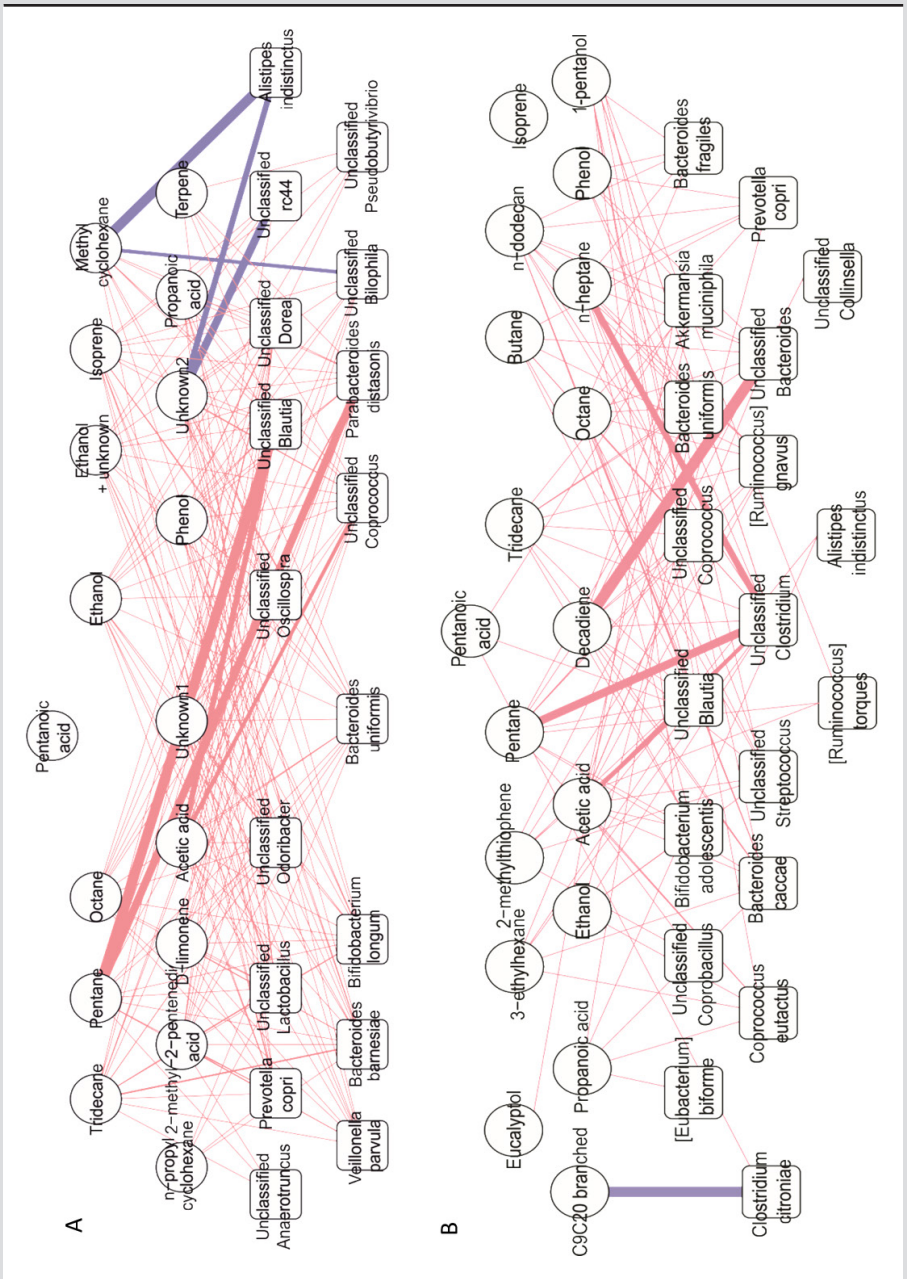
Nr.	Volatile metabolites	Nr.	Fecal bacterial taxa
1	Butane	1	***Bacteroides uniformis***
2	**Ethanol**	2	*Akkermansia muciniphila*
3	**Acetic acid**	3	*Ruminococcus gnavus*
4	Eucalyptol	4	***Prevotella copri***
5	Decadiene	5	*Bacteroides fragilis*
6	**Tridecane**	6	*Bacteroides caccae*
7	**Propanoic acid**	7	*Eubacterium biforme*
8	n-dodecan	8	*Coprococcus eutactus*
9	**Isoprene**	9	*Ruminococcus torques*
10	**Pentane**	10	*Alistipes indistinctus*
11	**Pentanoic acid**	11	*Bifidobacterium adolescentis*
12	n-heptane	12	*Clostridium citroniae*
13	1-pentanol	13	*Unclassified Collinsella*
14	**Octane**	14	*Unclassified Bacteroides*
15	3-ethylhexane	15	*Unclassified Streptococcus*
16	**Phenol**	16	*Unclassified Clostridium*
17	C_9C_{20} branched	17	***Unclassified Blautia***
18	2-methylthiophene	18	*Unclassified Coprococcus*
		19	*Unclassified Coprobacillus*

B



A) The chemical identification of the 18 breath metabolites and taxonomy of 19 faecal bacterial taxa that have the highest contribution to the correlation between volatile compounds in breath and faecal microbiome for active CD. Breath volatiles and faecal taxa that were identified as being among the most contributing to the correlation in both active and inactive CD samples are highlighted in bold. B) The Spearman correlation heatmap between 18 selected sets of breath metabolites and 19 bacterial taxa for active CD. The numbers in the heatmap correspond to names of the selected breath metabolites and faecal bacterial taxa in panel A. The colours in the heat maps correspond to the positive correlation for red and negative correlation for green. The white squares designate the significant correlation (p-value<0.05) between breath metabolites and faecal bacteria after Benjamini–Hochberg post-hoc correction.

Figure 5.5. Inter-omic Spearman correlation networks for A) CD in remission and B) active CD.



Pair-wise Spearman correlation was calculated between 17 faecal bacterial taxa and 17 breath metabolites for CD in remission (panel A) and 19 faecal bacterial taxa and 18 breath metabolites for active CD (panel B). All correlations with r<0.2 were removed. Blue edges represent negative correlation, while red edges represent positive correlation.

DISCUSSION

The development of next-generation sequencing platforms led to a major influx of studies on the gut microbiota in relation to numerous health related outcomes. Nevertheless, these studies often do not take into account functional read-outs or products of microbial metabolism, such as short chain fatty acids and metabolites of protein fermentation. This study represents the first effort to integrate faecal microbiome data with volatile metabolites in breath. For the current study, we chose to perform the analysis on two different stages of Crohn's disease (CD), *i.e.* active and remission. We decided to concentrate on CD since this disease is associated with both disturbance of exhaled breath content and perturbations in the bacterial composition in the gut [19, 20, 38-40]. We identified strong significant correlations between profiles of the volatile metabolites in breath and intestinal microbiota during active and inactive stage of the disease. This indicates that a major part of the exhaled volatile metabolites are directly or indirectly, *e.g.* by microbial metabolism of host metabolites, related to the microbiota composition. Interestingly, distinct correlations were found for samples collected in active and inactive CD, which may reflect underlying mechanisms. The CCA analysis revealed a slightly stronger correlation in inactive (R=0. 96) than in active disease (R=0.91). Moreover, a larger number of significant Spearman pair-wise correlations were observed in inactive than in active CD. Although, in both disease stages the CCA correlation between volatile metabolites in breath and faecal bacterial taxa was statistically significant, the majority of volatile metabolites and faecal bacterial taxa do not overlap between active and inactive disease. We found 9 overlapping volatile metabolites and only 3 faecal bacterial taxa between active and inactive disease. As shown in previous studies with CD patients, the profiles of exhaled breath and diversity of the faecal microbiota differ significantly between active and quiescent disease [19, 20]. Therefore, it is not surprising that the relation found here are also dependent on the disease stage. Another main result of this study was the rich network of important correlations between the bacterial taxa and volatile metabolites in breath. Such relation probably is caused by a combination of two general processes: 1) anabolism and catabolism of volatile metabolites by microbes, and 2) stimulation and inhibition of microbial growth by metabolites. Previous studies did show that major dietary changes (*i.e.* high protein and fat or high carbohydrates) result in alterations in gut microbiota composition and metabolic activity [9, 41]. Most of the pair-wise correlations between faecal bacteria and volatile metabolites in breath had a positive correlation coefficient, whereas only few of the observed correlations were negative.

In the current study, we found several SCFAs, acetic, pentanoic (valeric) and propionic acid, in exhaled breath contributing to the correlation with the faecal microbiota in active and inactive CD. Interestingly, the correlations were stronger and more statistically significant in remission stage of the disease than in active CD. SCFAs are formed during fermentation of non-digestible carbohydrates by colonic gut bacteria and have beneficial impact on host physiology as energy

source and by acting as signalling molecules [15, 42]. They have for example anti-inflammatory, anti-oxidative and anti-carcinogenic potential and are associated with a decreased risk of developing cancer, gastrointestinal disorders and cardiovascular disease. Acetate is the major SCFA in the colon, is rapidly absorbed and enters the general circulation. In the liver, after conversion into acetyl-CoA it enters the tricarboxylic acid cycle to be used as energy source [43], and as the main fatty acids. Acetate is also metabolised by muscle and other tissue, while propionate is primarily metabolised in the liver and can inhibit cholesterol synthesis. Colonic bacteria, specifically *Bifidobacterium* species, ferment oligosaccharides resulting in the generation of SCFAs [44-46]. Also members of the phylum Firmicutes are responsible for fermentation of carbohydrates and thus production of SCFAs. In the current study, we identified statistically significant correlations between acetate and propionate and *Bifidobacteria* and several members of the Firmicutes phylum in both remission and active stage of the disease. Additionally, *Blautia* showed a strong positive correlation to acetic acid and correlated moderately to propionate and pentanoic acid (*i.e.* valeric acid) in both active and inactive stage of CD. These findings are in agreement with the fact that *Blautia* are known to be major acetogenic bacteria in the human gut [47, 48]. Moreover, this bacterial species has been associated with disease activity in CD patients [19, 49]. Interestingly, the relative amount of acetic, valeric and propionate acids were reduced in active stage of the disease with respect to CD in remission. Similarly, the abundance of bacterial taxa correlating with these SCFAs, *i.e. Bifidobacteria, Blautia,* and members of the Firmicutes phylum were reduced in active stage with the disease.

We also found statistically significant correlations between *Bacteroides fragilis* and pentane and octane in active CD. *B. fragilis* belongs to a normal colonic commensal bacterial species found in the majority of adults [50]. Some of *B. fragilis* strains secrete a pro-inflammatory zinc-dependent metalloprotease toxin that is associated with diarrheal diseases. These strains have been identified in active IBD [51]. The set of volatile metabolites that is correlated to *B. fragilis*, are mostly formed during the process of reactive oxygen species (ROS) induced lipid peroxidation and thus associated with inflammation [52, 53]. Similarly to *B. fragilis* another bacterial species, *Ruminococcus gnavus*, also revealed statistically significant correlations to volatile metabolites that are formed during ROS. *R. gnavus* is a commensal mucin-degrading bacterium. This bacterial species was found to be overrepresented in IBD in comparison to healthy controls and has been reported to increase intestinal permeability [54]. These correlations do not necessarily imply that these bacteria produce these metabolites, but as the bacterial abundance and VOC concentrations can also share a common cause. In the current study, phenol correlated significantly to several *Bacteroides* species and *Provotella copri* in the active stage of CD. Kumar *et al.* showed that phenol is produced by tyrosine metabolism by gut bacteria [55]. More specifically, several bacterial species including Bacteroides were associated with phenol production [56]. The relation of phenol to *P. copri* has not been demonstrated previously. It has been shown to have the potential to break down the intestinal barrier and to trigger proinflammatory events

in arthritis [57, 58]. Although, our results are in agreement with previous findings, we should be aware that the phenol detected in the current study is the sum of phenol produced by gut bacteria, but also some amount of phenol that may come directly from the Tedlar bags used to sample exhaled breath.

The combined analysis of volatile metabolites in breath and faecal bacteria may offer several beneficial aspects. First of all, the joined analysis may enable better description of microbiota-related chronic diseases, such as CD. Moreover, pooling potential biomarkers from different sources such as breath metabolites and faecal bacteria, may improve the power of disease diagnosis. Another benefit is better understanding of the underlying processes by integrated analyses of the microbiota composition and breath volatile metabolites. This study demonstrates for the first time a strong correlation between the profiles of the intestinal microbiota and volatiles in exhaled breath, which is partly dependent on CD disease stage. Irrespective of the mechanism of correlation, these strong relations have several potential implications for future innovations, for instance, strong correlation may be used as biomarker for individual or groups of analytes. Moreover, information on the relation between volatile metabolites in breath and faecal microbiome may be valuable in therapeutic or dietary interventions for chronic disorders with associated microbial perturbations.

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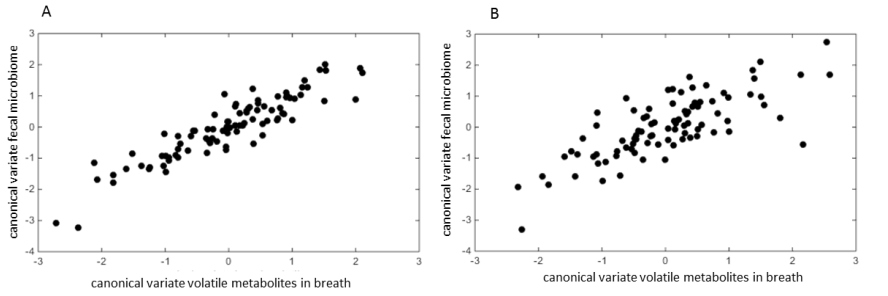
SUPPLEMENTAL INFORMATION

S5.1. The canonical correlation score plot using the first canonical variates of 650 volatile metabolites in breath (x-axis) and 6629 OTUs (y-axis)) for: A) inactive CD patients; B) CD in active stage.



Each point in the figure represents the combined information of a single breath and faecal sample collected at a single follow-up moment from the same CD patient. In both plots there is clear structure in the presented data. The CCA analysis revealed significant correlation between volatiles in exhaled breath and faecal bacterial taxa with R=0.85 and p-value 0.0015 for CD in remission and R=.0.82 with p-value 0.001for active CD. The first canonical correlation coefficient explains 72% of variance for patients in remission and 67% for active CD.

6

The impact of sleeve gastrectomy on the fecal microbiota of obese individuals

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ABSTRACT

Background: Emerging evidence indicates that the intestinal microbiota plays a role in the etiology of obesity. Bariatric surgery results in significant weight loss and is often a last resort to combat obesity and its associated co-morbidities. The sleeve gastrectomy procedure (SG) is increasingly used, but its effect on the intestinal microbiota and obesity-related complications has not been investigated as thoroughly as for other bariatric procedures (*e.g.* Roux-en-Y Gastric Bypass).

Objective: To investigate the effect of sleeve gastrectomy (SG) on the fecal microbiota of obese individuals and to examine if shifts in the microbiota are associated with markers of inflammation, intestinal barrier function and glycemic control.

Methods: Fecal and blood samples of healthy individuals (n=27) and obese individuals pre-SG (n=24), two months (n=13) and six months post-SG (n=9) were collected. The V4 region of the 16SrRNA gene was sequenced by the Illumina MiSeq method to assess the microbiota composition. Furthermore, fecal calprotectin, and plasma hsCRP and Hba1c were determined as well as small and large intestinal permeability by a multi-sugar test.

Results: The fecal microbiota composition between obese and lean individuals was significantly different. These differences did correlate to BMI but not to other host factors. The fecal microbiota composition two and six months post-SG was significantly different, *i.e.* characterized by an increase of *Streptococcus, Actinomyces* and *Rothia* and a loss of *Dialister,* as compared to the microbiota composition prior SG (p=0.008), but did not change towards a more lean profile. Although hsCRP and Hba1c decreased significantly post-SG, no correlations with microbial alterations were found with these, nor with fecal calprotectin or intestinal permeability markers.

Conclusion: The fecal microbiota composition of patients with obesity changed significantly following SG, but not towards a lean microbiota composition. The post-SG microbiota was characterized by an increase of facultative anaerobic bacteria, characteristic for the upper gastrointestinal tract, which might be explained by the physiological changes introduced by the SG procedure. More long-term observational as well as mechanistic studies are warranted to investigate the persistence of the observed microbial changes and to what extend these changes impact host physiology and metabolic improvements.

INTRODUCTION

Obesity is an emerging public health problem [1], associated with co-morbidities such as cardiovascular disease, type 2 diabetes and non-alcoholic fatty liver disease [2], and has substantial impact on direct healthcare as well as indirect costs (*e.g.* due to loss of work productivity) [3]. Obesity is related to western lifestyle, characterized by an increased intake of energy-dense foods and a reduction of physical activity. Emerging evidence indicates that also the intestinal microbiota plays a role in the etiology of obesity [4,5]. First evidence provided by Turnbaugh *et al.,* showed that the microbiota of genetically leptin-deficient obese mice had an increased capacity to extract energy from the diet when compared to that of lean wild-type mice. Moreover, the obese phenotype was found to be transmissible by transplantation of the cecal contents of obese donor mice into germ-free lean recipients [6]. In line with these findings, transplanting the microbiota of obese humans to adult germ-free mice also resulted in increased total body and fat mass, as well as obesity-associated metabolic phenotypes [7]. An increased Firmicutes/Bacteroidetes ratio [8–10] and a reduced microbial diversity has repeatedly been reported in obese as compared to lean individuals [11], but has not been confirmed in all studies [12–14]. From a mechanistic point of view, the microbiota may promote weight gain, *e.g*. by increasing energy harvest via the production of short chain fatty acids (SCFAs), and by influencing the expression of genes that promote fat storage satiety (*e.g. ANGPTL4*) [15–17]. Furthermore, intestinal bacteria can affect the immune system and expression of tight junctions, promote bacterial translocation and contribute to inflammation and obesity-related complications (*e.g.* non-alcoholic fatty liver disease and type two diabetes) [15,17–20].

As life style modifications such as caloric restriction and exercise are often not successful, surgical interventions are the last resort for weight loss and reducing obesity-related comorbidity [21–23]. Roux-en-Y gastric bypass (RYGB) is a frequently applied bariatric procedure with good long-term outcome [24]. RYGB results in a small gastric pouch and bypasses the distal stomach, duodenum and proximal jejunum and is characterized by reduced acid secretion, increased transit, alterations in the enterohepatic cycle and GI hormone secretions [25]. Post-bariatric surgery alterations in the microbiota have been observed, including an increased microbial diversity and altered composition, especially an increase of P*roteobacteria* and *Akkermansia,* and decrease of Firmicutes [8,26–29]. Whether these alterations are due to weight loss, altered dietary intake, or procedural consequences on GI physiology is unclear. Furthermore, Zhang *et al* showed that the fecal microbiota does not change towards a more lean profile [26]. Nowadays, use of sleeve gastrectomy (SG) is emerging, being less invasive with promising mid- and long-term efficacy [24,30]. To the best of our knowledge, only one human study has investigated the effect of SG on the fecal microbiota comparing five subjects post-SG with five subjects undergoing a standardized caloric restricted diet, showing an increase of Bacteroidetes and decrease of Firmicutes six months post-surgery [31]. More studies on the effect of SG on the

fecal microbial composition are needed. Furthermore, it would be of importance to evaluate whether post-operative changes result in a shift towards that of lean subjects and are associated with resolution of markers for obesity-related complications. Within the present study, we therefore aimed i) to gain insight into the effect of SG on the fecal microbiota; and ii) to examine whether shifts in the microbiota are associated with markers of inflammation, intestinal barrier function, and glycemic control.

MATERIAL AND METHODS

Morbidly obese subjects

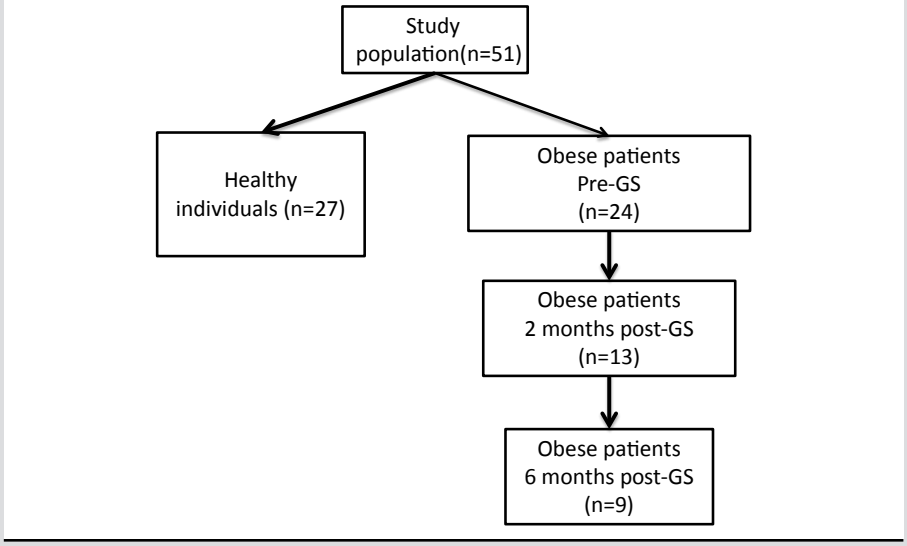
Obese individuals undergoing a SG in the Catherina hospital in Eindhoven, The Netherlands in 2011 with a stable weight for at least three months prior to surgery, were asked to participate in the current study. In total, 24 obese individuals between 18-65 years of age with either a BMI>40 kg/m^2 or a BMI>35 kg/m^2 in combination with comorbidities (*i.e.* diabetes and/or hypertension) were included. Exclusion criteria were severe hematological, immunological, neurological, psychiatric, respiratory or cardiovascular diseases, gastrointestinal or hepatic disorders influencing gastrointestinal absorption, transit and/or microbiota, alcohol consumption >20 units/week, eating disorders, blood donation less than 3 months prior to study participation, pregnancy, lactation, use of antibiotics less than 4 weeks prior to study participation and previous gastro-intestinal surgery (except appendectomy and cholecystectomy). Subjects undergoing SG received a preoperative diet (Modifast (500kcal), Breda, The Netherlands) for two weeks and per-operative antibiotic prophylaxis (single dose of 1000 mg cefazolin). Two months prior to SG (T1), intestinal permeability was determined by a multi-sugar test, and fecal and blood samples were collected from all 24 patients [32]. A subgroup of patients was willing to repeat the intestinal permeability test and collection of fecal and blood samples two (T2; n=13) and six months (T3; n=9) post-SG (figure 6.1). All study subjects were requested to refrain from alcohol consumption and to stop proton pump inhibitors (PPI) and NSAID use one week prior to each sampling day. Demographics, medication use and clinical data, were collected using a standardized questionnaire filled out by the treating physician.

Control subjects

27 age- and gender matched healthy lean subjects with a BMI ranging from 18 to 25 were recruited by public advertisements, within the context of related studies on the microbiota and intestinal permeability, and will further be referred to as lean subjects [33,34]. Exclusion criteria were identical to those for obese individuals. Fecal and blood samples were collected using the same standardized protocol as for the obese individuals.

The obese and lean subjects included in this study gave written informed consent prior to participation. The studies from which patients undergoing SG and lean control subjects were included, have been approved by the Medical Ethics Committee of Catharina Hospital Medical Centre (for obese subjects; M11-1109 (CCMO NL34712.060.11)) and the Maastricht University Medical Center+ (for lean subjects; CCMO NL24160.068.08 and CCMO NL NL31078.068.09) and were executed according to the revised declaration of Helsinki (59th general assembly of WMA, Seoul, South Korea, October. 2008).

Figure 6.1. Flowchart of study design



Feces and blood collection and determination of intestinal permeability

Fecal samples were frozen at -80 ºC within 12 hours of defecation until further analysis. To correct for differences in consistency, fecal protein concentrations were determined by the Pierce BCA Protein Assay kit (ThermoFisher scientific, Waltham, USA) as described previously [35]. Fecal calprotectin levels were determined by ELISA (Bühlmann Laboratories, Switzerland) according to the manufacturer' instructions. Blood samples were sent to the laboratory of Clinical Chemistry for routine analysis of hsCRP and Hba1c in plasma.

The multi-sugar test with 24-hour urine collection was performed to assess gastrointestinal permeability in both obese and control subjects as described previously [33]. The lactulose to rhamnose ratio (L/R) in 0-5 hour urine fractions and the sucralose to erythritol ratio (S/E) in 5-24 hour urine fractions were used as indicators for small and large intestinal permeability, respectively. Sucrose excretion in 0-5 hour urine was used to determine gastroduodenal permeability.

DNA isolation of fecal samples

A portion of approximately 200 mg was obtained from frozen fecal samples by cutting on ice to prevent thawing of samples. Subsequently, DNA was isolated using a combination of bead beating and column-based purification with the PSP spin stool kit (Stratec Molecular, Berlin, Germany) as described previously [36]. PCR-grade water was used as negative control. DNA concentrations were determined by Quant-IT Pico Green dsDNA reagent kit (Invitrogen, New York, USA) using the Victor3 Multilabel Counter (Perkin Elmer, Waltham, USA).

Sequencing

The V4 region of the 16S rDNA gene was amplified using the 515f/806r primer as described previously [37]. The PCR reaction was performed using 25 µl NEB Phusion High-Fidelity Master Mix (New England Biolabs, Ipswich, USA), 4 µL 515f/806r primer mix and 30 ng metagenomic DNA under the following conditions: denaturation at 98°C for 3 minutes, followed by 30 cycles of denaturation at 98°C for 45 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 45 seconds. The final elongation step was at 72°C for 7 minutes. Amplicons were purified by using AMPure XP purification (Agencourt, Massachusetts, USA) according to the manufacturer's instructions, mixed in equimolar concentrations and sequenced on an Illumina MiSeq instrument.

Data analysis

Subject characteristics are presented as median (interquartile range) for continuous variables and as absolute numbers (proportions) for categorical variables (table 6.1). To test for differences between healthy and obese subjects, the nonparametric Mann-Whitney U test and the X^2 test were used for continuous and categorical variables, respectively, whereas the paired nonparametric Wilcoxon signed rank test and McNemar test were used to test for changes within obese subjects over time.

Sequences were analyzed using a workflow based on QIIME 1.8 [38]. Operational taxonomic unit (OTU) clustering, taxonomic assignment and reference alignment were done with the pick_open_reference_otus.py workflow script of QIIME, using Usearch as clustering method (97% identity) and the GreenGenes database v13.8 as the reference database [39]. Reference-based chimera removal was done with UCHIIME [40].

To examine the microbial richness and diversity within samples, the following alpha-diversity indices were calculated: Chao1, Shannon index and PD whole tree. The Mann-Whitney U test (MWU) was used to analyze significant differences between lean and obese subjects, and the Wilcoxon signed rank test was used to analyze significant changes within obese individuals over time (T1 vs. T2, T1 vs. T3 and T2 vs. T3).

Principal components analysis (PCA) was used to visualize the microbiota structure between the different groups. Subsequently, partial redundancy analysis (RDA) was applied to analyze the microbiota structure between lean and obese individuals, and obese individuals before and after SG. In this partial RDA, we included the variable "subject" as a covariate to remove the variation explained by the repeated measurements. A list of the 20 most important taxa for the separation and their corresponding importance score can be found in supplemental table 6.1, 6.2, 6.3 and 6.4. To detect potential confounding by diabetes, smoking, medication use, gender and fecal consistency, RDA (for diabetes, smoking, medication use and gender) and Canonical

Correspondence Analysis (CCA) (fecal consistency) were performed to examine whether any of these variables were associated with the microbiota composition in lean and/or obese subjects, respectively. CCA and RDA were also used to investigate whether associations were present between the microbiota and markers for inflammation (fecal calprotectin and plasma hsCRP), intestinal barrier function (urinary sucrose, L/R and S/E ratio) and glycemic control (Hba1c). PCA, RDA and CCA were performed in Canoco version 5. The Wilcoxon signed rank test was used to find significant differences in the relative abundances of specific taxa between T1, T2 and T3. To correct for multiple testing, the Bonferroni corrected p-value threshold of 0.05 was used. The fold increase or decrease of specific taxa between pre-SG and post-SG was calculated by dividing the relative abundance in post-SG by the relative abundance in pre-SG. The potential relationships between the taxa that were most strongly affected by SG in obese patients during follow-up and hsCRP, calprotectine, Hba1c and intestinal permeability markers were investigated using the Pearson product-moment correlation analysis. To correct for multiple testing, the False Discovery Rate method was used. A cut-off value of 0.05 was used.

RESULTS

Study population

Clinical characteristics of lean and obese individuals are given in table 6.1. None of the included subjects used probiotics or antibiotics during the study, except for the prophylaxis given for the SG procedure. BMI was significantly lower in lean as compared to obese individuals prior to ($p=9.6\cdot10^{-10}$), but also two ($p=8.14\cdot10^{-7}$) and six months ($p=8.94\cdot10^{-6}$) after SG. Within the obese group, SG resulted in a significant BMI loss two ($p=2.21\cdot10^{-3}$) and six months post-surgery ($p=7.69\cdot10^{-3}$). Hypertension and type 2 diabetes were present in 58.3% and 41.7% of obese subjects, respectively, while absent in the control subjects. Two and six months post-SG, Hba1c decreased significantly (p=0.011 and p=0.018, respectively), though none of the subjects with follow-up data did obtain resolution of diabetes. Furthermore, two obese individuals (22%) were able to obtain resolution of hypertension following SG.

Inflammation markers hsCRP in plasma and fecal calprotectin were significantly lower in the lean as compared to the obese subjects. Moreover hsCRP decreased within the obese individuals between baseline and six months post-SG. No decrease was found for fecal calprotectin post-SG.

Urinary sucrose excretion, but not the L/R and S/E ratios, was significant higher in the obese (T1) versus lean individuals. Post-SG, L/R ratio decreased significantly after six months (T3) and the S/E ratio decreased significantly after two months (T2) when compared to baseline (T1) within the obese group. Fecal protein content did not significantly differ between groups.

Table 6.1. Clinical characteristics of the study population

	Lean subjects (n=27)	Obese subjects T1 (n=24) pre-SG	T2 (n=13) two months post-SG	T3 (n=9) Six months post-SG
BMI (median, IQR)	23.0 (21.5-24.4)*	43.5 (39.4-48.3)*$	37.9 (36.0-43.0)*$	34.8 (31.5-39.4)*$
BMI loss (%) from baseline (median, IQR)	Not applicable	Not applicable	15.8 (11.9-20.2)$	23.5 (21.4-27.8)$
Age (median, IQR)	47 (24-61)	43 (37-55)	50 (41.5-55)	50 (42-55)
Male (n, %)	12 (44.4)	11 (45.8)	7 (53.8)	5 (55.6)
Current smokers (n, %)	3 (11.1)	7 (29.2)	3 (23.1)	3 (33.3)
Hypertension (n, %)	0 (0)#	14 (58.3)#	9 (69.2)#	5 (55.6)#
Type 2 diabetes (n, %)	0 (0)#	10 (41.7)#	6 (46.2)#	4 (44.4)#
Medication (n, %)				
Insulin	0 (0)#	4 (16.7)#	3 (23.1)	0 (0)
Oral antidiabetics[1]	0 (0)	5 (20.8)	2 (15.4)	4 (44.4)
Metformin[2]	0 (0)	8 (33.3)	5 (38.5)	4 (44.4)
PPI use (%)[3]	1 (3.7)	2 (3.8)	3 (23.1)	1 (11.1)
Antibiotics[4]	0 (0)	0 (0)	0 (0)	0 (0)
Past antibiotics[5]	1 (3.7)#	0 (0)	0 (0)	1 (11.1)

Table 6.1 (continued). Clinical characteristics of the study population

	Lean subjects (n=27)	Obese subjects T1 (n=24) pre-SG	T2 (n=13) two months post-SG	T3 (n=9) Six months post-SG
Plasma markers				
hsCRP (mg/l, median, IQR)	0.6 (0.4-0.9)*	6.3 (3.0-4.0)*$	7.3 (5.4-11.2)*&	3.4 (1.9-4.1)*$&
Hba1c (mM Hba1c/M HB median, IQR)	Not available	49 (40-73)$	41 (35-55) $	43 (33-53) $
Fecal markers				
Calprotectin(µg/gram feces, median, IQR)	1.4 (0.8-3.4)*	3.7 (2.0-9.7)*$&	15.6 (11.9-21.0)*$	10.7 (8.6-13.7)*&
Urinary markers				
Sucrose (µg, median, IQR)	1958.8 (1042.8-3716.6)*	5642.1 (1956.3-24273.7)*	1846.2 (450.7-4565.1)	2758.5 (1174.0-5694.4)
Lactulose/rhamnose (median, IQR)	0.04 (0.03-0.08)	0.05 (0.02-0.07)$	0.05 (0.03-0.06)&	0.045 (0.04-0.10)$&
Sucralose/erythritol (median, IQR)	0.06 (0.02-0.08)	0.05 (0.02-0.06)$	0.08 (0.03-0.09)$	0.04 (0.02-0.06)
Protein content (µg protein/mg feces, median, IQR)	117.4 (92.2-192.5)	127.0 (68.5-200.6)	162.6 (52.7-237.6)	162.2 (101.2-231.4)

[1] sulphonylurea derivates and thiazolidinedione
[2] Oral medication
[3] Stopped 1 week prior fecal sampling
[4] Antibiotic use at moment of sampling
[5] Antibiotics use between 8 and 4 weeks prior sampling moment

* p<0.05 according to Mann Whitney U test HC vs T1; HC vs T2; HC vs T3
# p<0.05 according to chi squared test between HC vs T1; HC vs T2; HC vs T3
$ and & p<0.05 according to Wilcoxon signed rank test. Identical symbol indicates significant differences between 2 groups.

Sequencing data

A total of 3,267,977 raw sequences were obtained from 73 samples (n=27 HC, and n=24 obese T1, n=13 T2 and n=9 T3). After quality filtering and binning, 2,405,726 sequences with a median of 29,867 sequences per sample (IQR: 26,611 35,723) were available for downstream analysis.

The fecal microbiota of healthy individuals and obese patients

The microbiota composition at phylum level of lean individuals and obese patients pre- and post-SG is shown in figure 6.2. Prior to surgery, obese individuals, had a significant lower relative abundance of Bacteroidetes (15.1% vs 36.4%, resp., $p=9.5*10^{-7}$), Proteobacteria (1.8% vs 3.2%, resp., $p=2.1*10^{-4}$) and Tenericutes ($1.3{\cdot}10^{-3}$% vs $7.6{\cdot}10^{-3}$%, resp., $p=91.0*10^{-3}$) as compared to lean individuals. Firmicutes (70.5% vs 55.5% resp., $p=1.2*10^{-4}$) and Actinobacteria (0.1% vs 0.03%, resp., $p=1.2*10^{-7}$) were significantly higher in obese patients prior to SG as compared to lean individuals. In line with this observation, the Firmicutes/Bacteroidetes (F/B) ratio was significant higher in the obese as compared to lean individuals ($p=3.00*10^{-5}$, supplemental figure S6.1). At both two ($p=1.51*10^{-7}$) and six ($p=8.00*10^{-6}$) months after SG, the F/B ratios of the obese patients remained significantly higher as compared to the lean individuals (supplemental figure S6.1).

When examining the microbiota within the group of obese individuals over time, changes in the relative phyla abundances could neither be observed at two nor at six months post-SG as compared to baseline.

The richness and diversity of the fecal microbiota of lean and obese individuals

When compared to the lean individuals, the microbiota of individuals with obesity was characterized by a significantly lower microbial richness, as indicated by the number of observed species (median [IQR]: 1529.6 [IQR: 1351.6-1735.6] vs 1280.3 [1079.4-1631.9]; p=0,02). The richness as estimated by the Chao1 index was also lower, but not statistically significant. Moreover, the microbial diversity, taking into account both microbial richness and evenness, was also significantly lower in obese individuals when assessed by the Shannon diversity index (6.8[6.0-7.5] vs 7.5 [7.0-7.9]; p=0.02) as well as by the phylogenetic diversity metric PD whole tree (74.1[61.4-91.0] vs 85.0 [77.2-95.0]; p=0.02) (figure 6.3). Two and six months post-SG, the microbial richness and diversity of obese patients was still lower as compared to lean individuals, but this difference was no longer significant.

The fecal microbiota structure of lean and obese individuals.

Clustering of fecal samples by means of PCA indicated that the fecal microbial community structure of lean individuals could be clearly separated from that of obese patients at baseline (pre-SG). Furthermore, the fecal microbiota composition of obese patients two and six months post-SG tended to cluster apart from the microbiota of obese patients pre-SG , but did not change towards that of lean individuals (figure 6.4).

To further investigate the baseline differences in microbiota composition of obese and lean individuals, a constrained ordination method, RDA, was performed. RDA confirmed the separation of fecal samples by health status and showed that BMI significantly contributed in explaining the variation in microbiota composition (variance explained = 12.2%, Monte Carlo permutation test: p=0.002) (figure 6.5). This separation was driven by a higher relative abundance of the genera *Odoribacter* (MWU; Bonferroni corrected, $p=7.35 \cdot 10^{-5}$), *Bacteroides* ($p=4.11 \cdot 10^{-4}$), *Sutterella* ($p=2.14 \cdot 10^{-5}$), *Lachnospira* ($p=2.04 \cdot 10^{-5}$), *Butyricimonas* ($p=2.02 \cdot 10^{-5}$) and *Parabacteroides* (p=0.04) in lean subjects and a concomitant higher relative abundance of *Eubacterium* (p=0.02), *Dorea* ($p=5.13 \cdot 10^{-3}$) and *Ruminococcus* ($p=2.56 \cdot 10^{-3}$) in obese individuals (figure 6.5). A list of the 20 most important taxa for the separation and their corresponding importance score can be found in supplemental table 6.1. Including "BMI" as a covariate to remove the variation introduced by BMI, the partial RDA showed that gender, age, smoking, metformin, antidepressants and PPI use did not significantly contribute to the separation of the fecal samples (variance explained = 21.2%, p=0.31).

Figure 6.2. Visual representation of the microbiota composition in healthy, lean individuals (a) and obese patients pre-GS (b), two (c) and six months (d) post-GS.

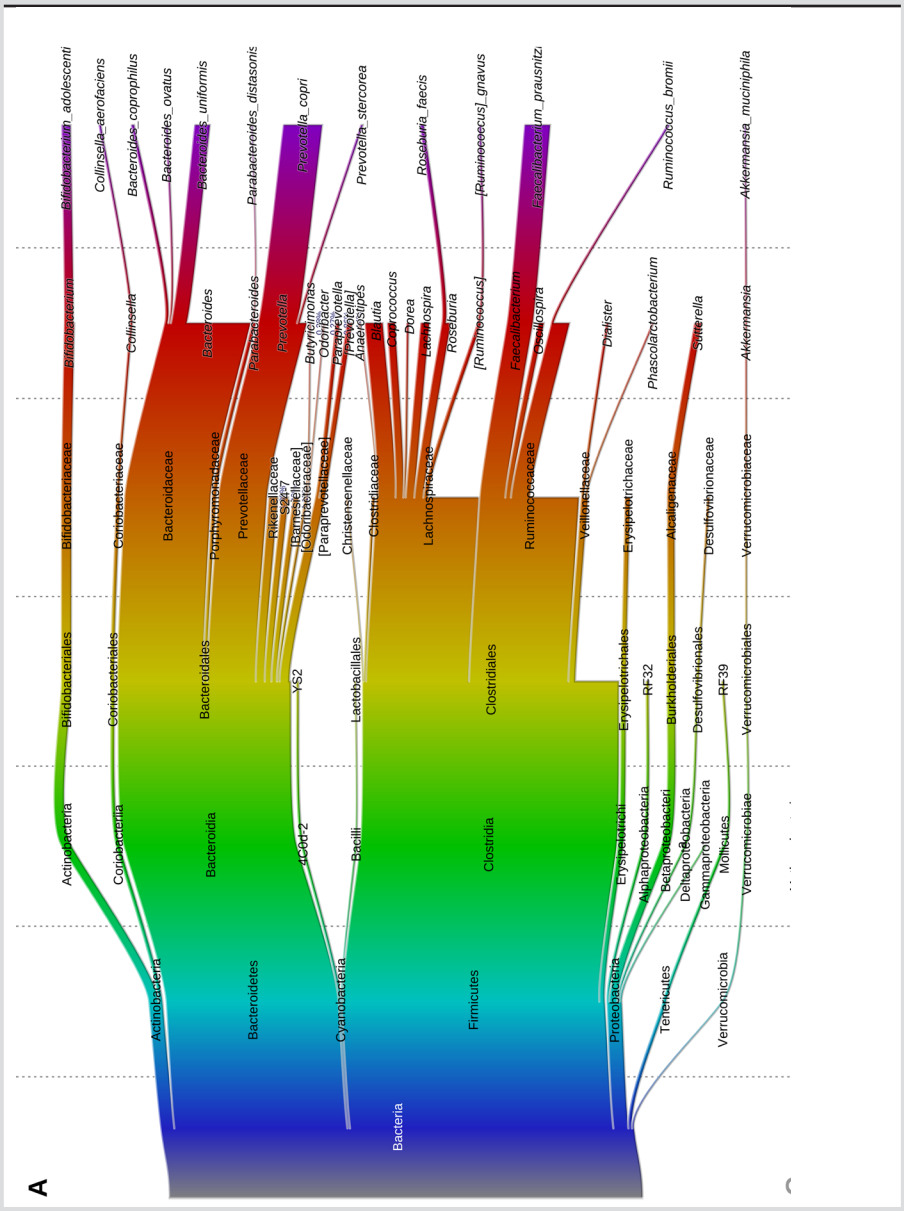


Figure 6.2. Continued.

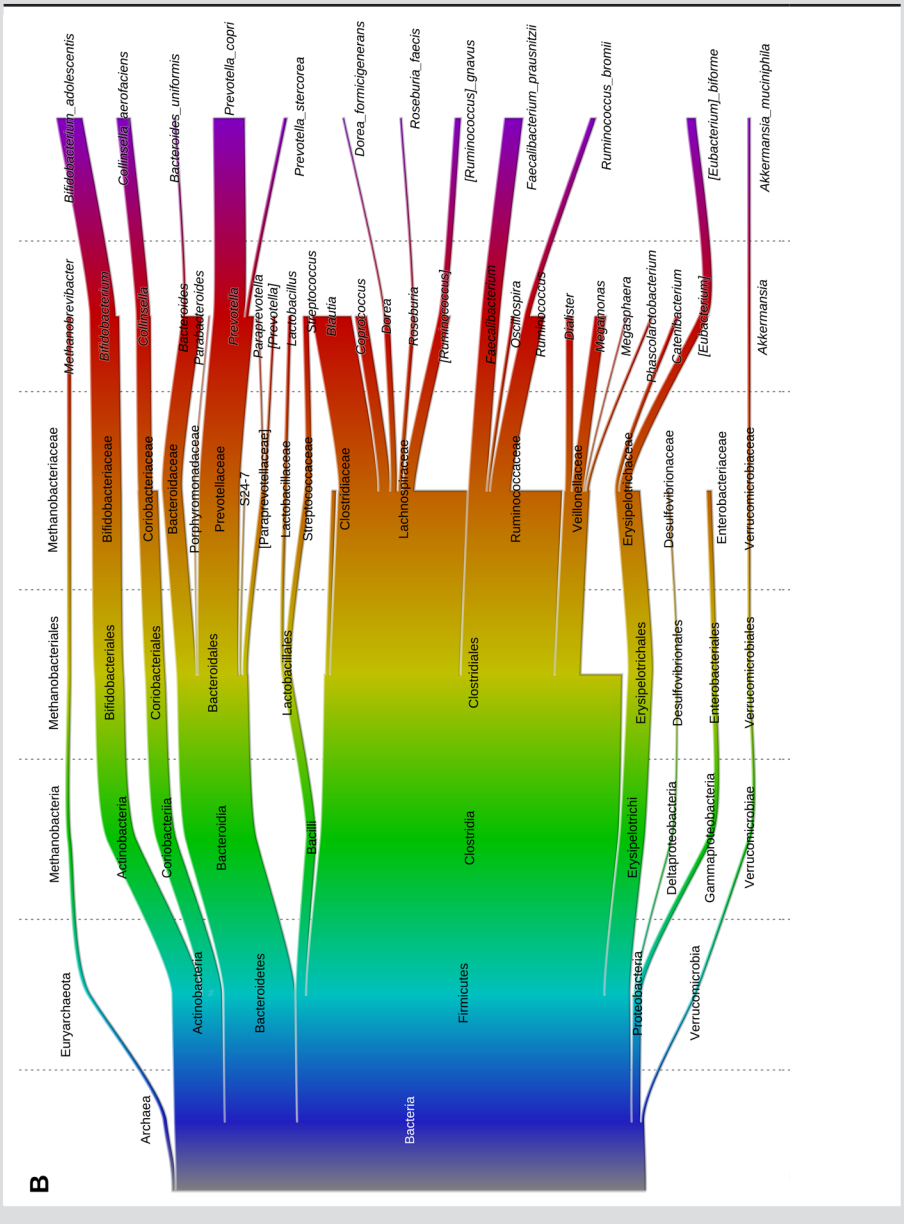


Figure 6.2. Continued.

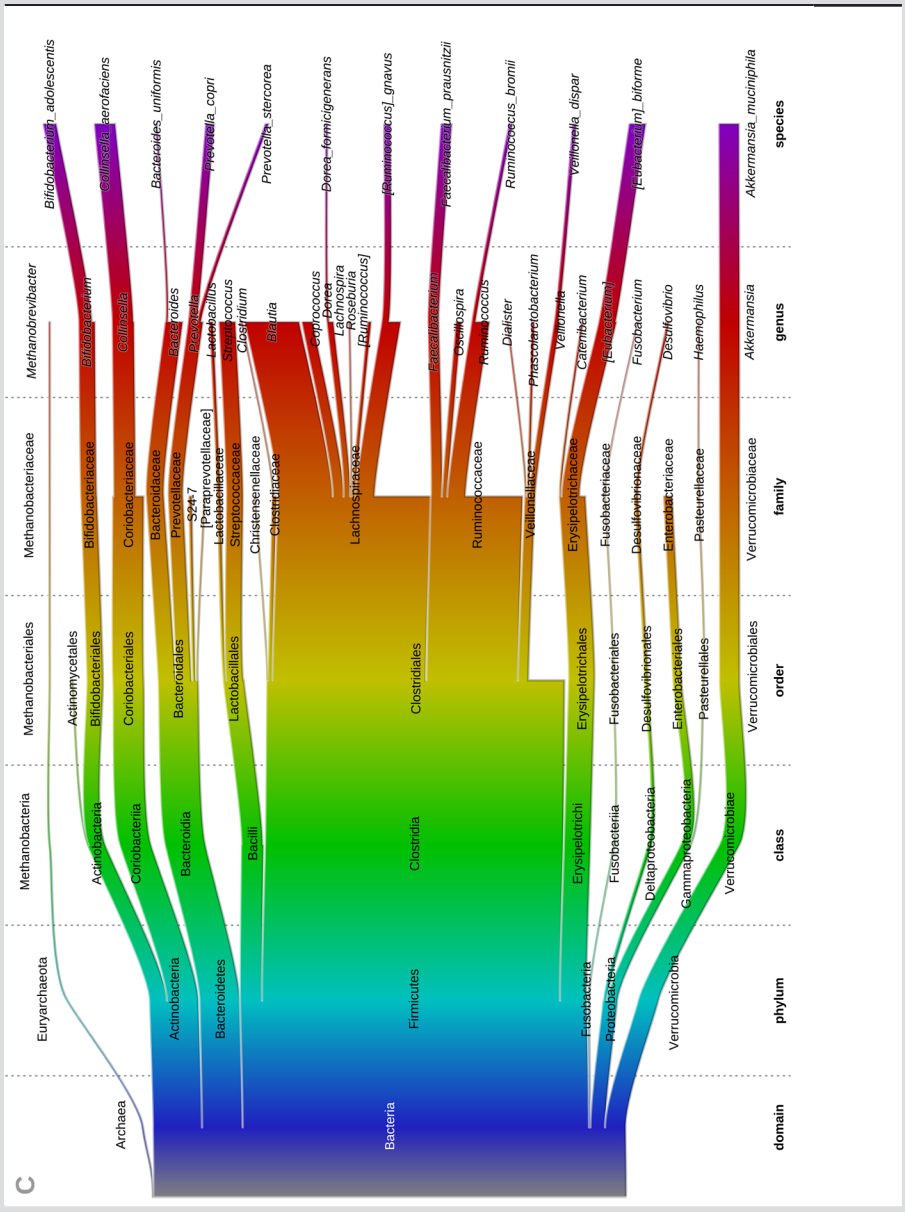
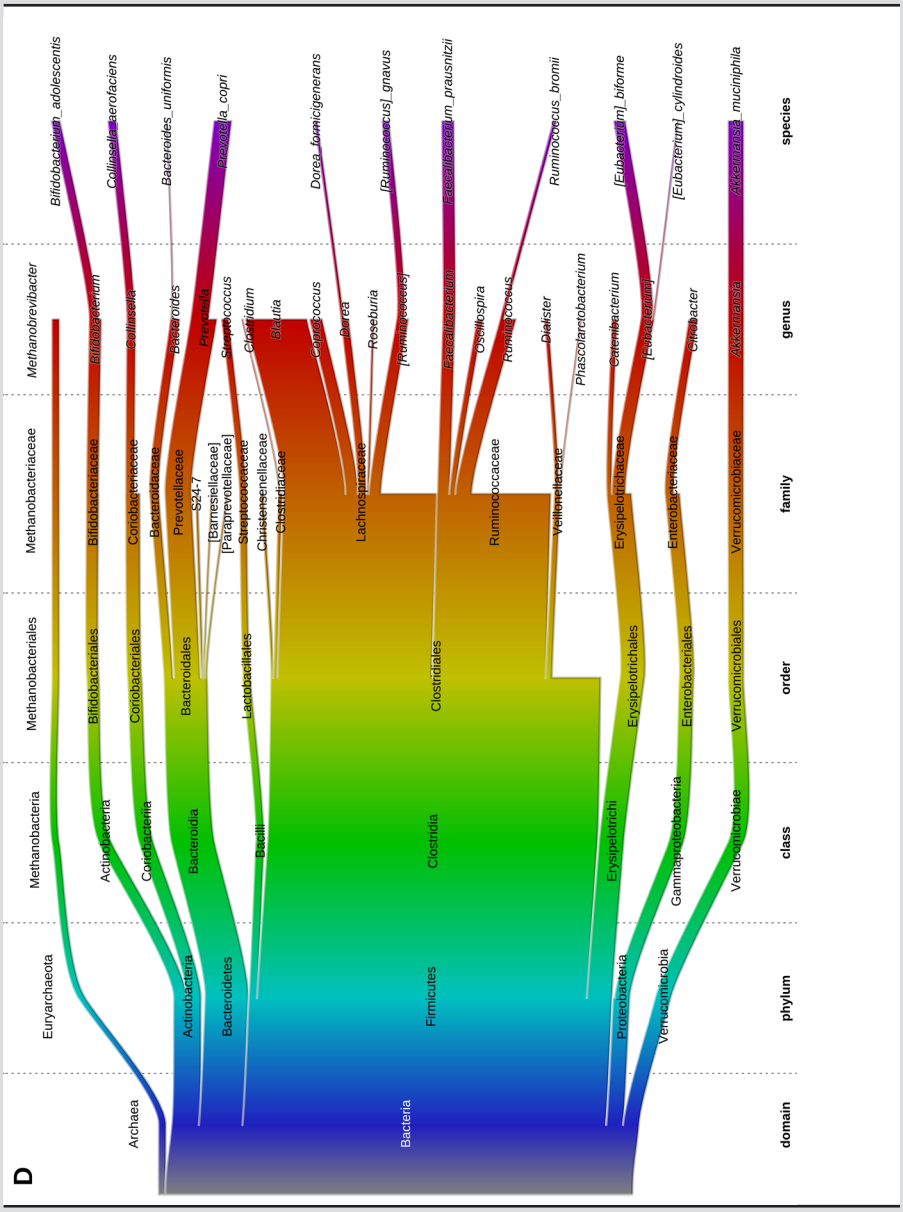
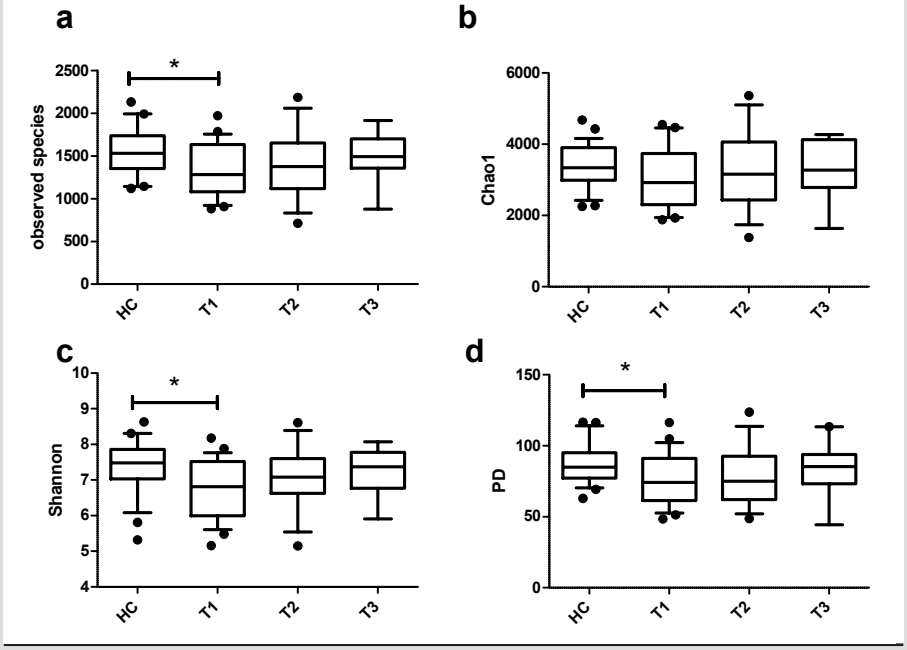


Figure 6.2. Continued.



The microbiota is presented from the domain to the species level.

Figure 6.3. Alpha diversity indices (observed species (a), chao1 (b), Shannon diversity index (c) and PD whole tree (d) of healthy individuals (HC) and obese patients pre (T1), two (T2) and six months (T3) post-SG.

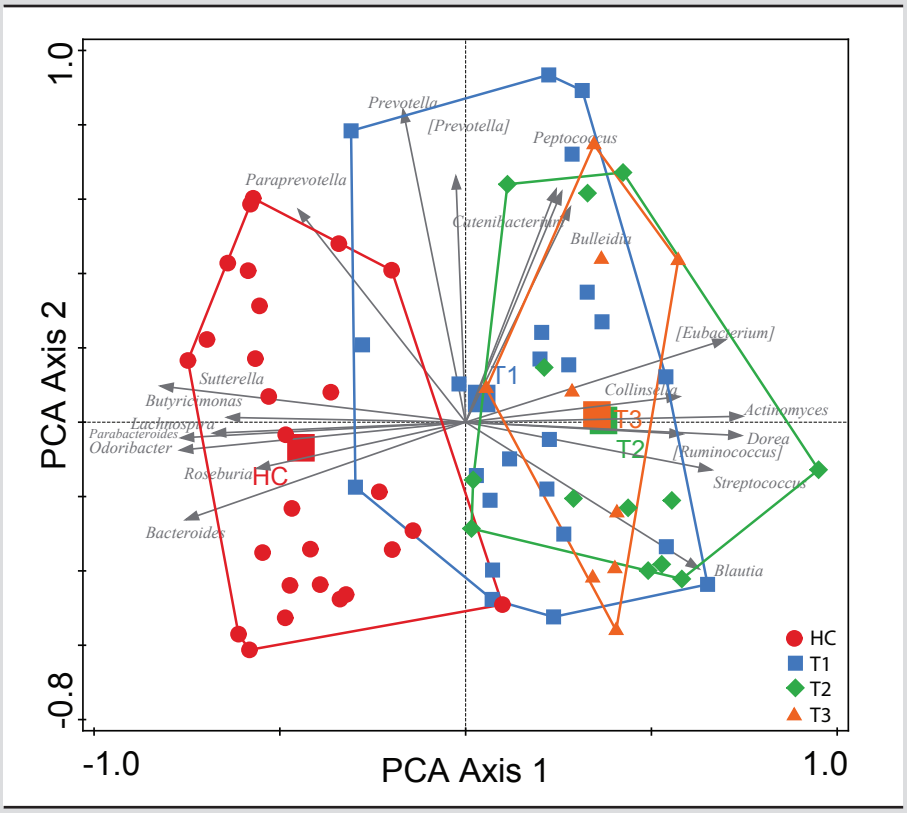


Data are presented as boxplots displaying medians with interquartile ranges. *indicates p<0.05

Effect of the gastric sleeve procedure on the fecal microbiota in obese individuals

To further investigate the fecal microbiota change within the obese individuals post-SG, a partial RDA was conducted in which the variable "subject" was included as a covariate to remove variation introduced by host specificity. Partial RDA on the fecal samples of obese individuals pre- and post-SG, indicated that the fecal microbiota changed significantly following SG (variance explained: 19.6%, p=0.008) (figure 6.6). The separation was mainly driven by a decreased abundance of the genus *Dialister* and an increased abundance of *Streptococcus, Actinomyces* and *Rothia* in post- as compared to pre-SG (supplemental figure S6.2). A list of the 20 most important taxa for the separation and their corresponding importance score can be found in supplemental table 6.2.

Figure 6.4. PCA based on the fecal microbiota composition of healthy individuals (red circle), obese individuals pre (T1; blue squares), two (T2; green diamonds) and six months (T3; orange crosses) post-SG.

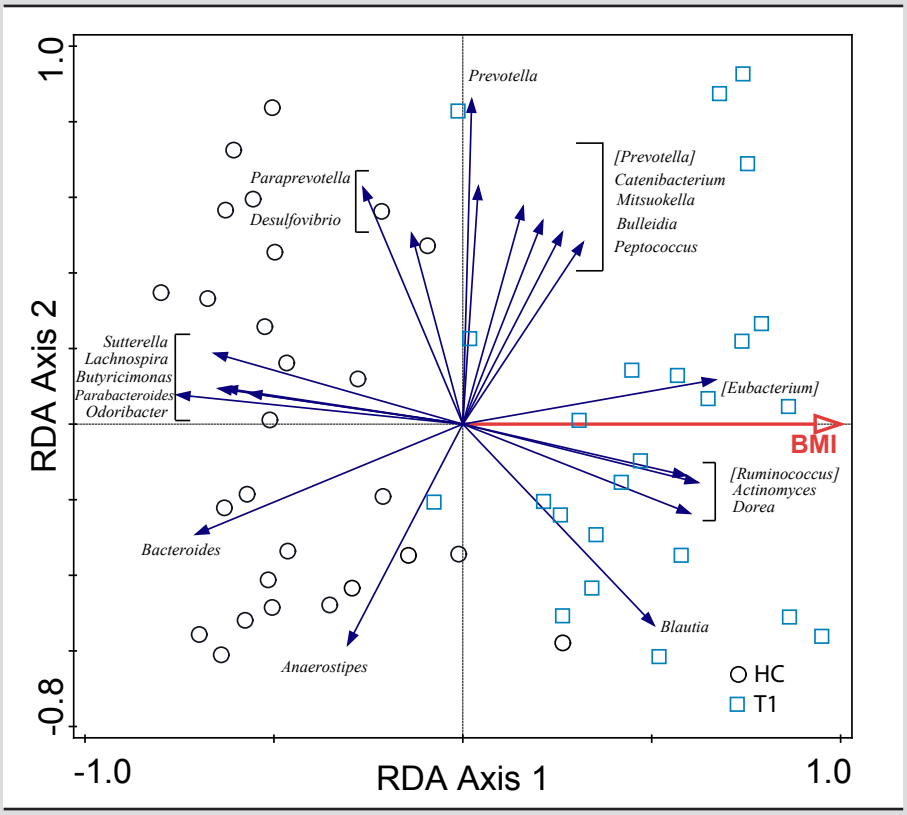


To further elucidate the shifts in specific bacterial taxa in obese subjects following SG, the 20 top-classifier bacterial taxa from the partial RDA were selected and the changes in the relative abundance of these taxa were analyzed with the Wilcoxon signed rank test. Of those signature genera, *Streptococcus, Actinomyces, Rothia,* were significantly increased both at two (fold increase of 14.6; $p=1.8 \cdot 10^{-3}$, 4.6; $p=3.0 \cdot 10^{-3}$ and 24.5; $p=3.7 \cdot 10^{-3}$ respectively) and six months (fold increase of 11.7; $p=7.7 \cdot 10^{-3}$, 2.8; $p=7.7 \cdot 10^{-3}$ and 7.0; $p=7.7 \cdot 10^{-3}$ respectively) post-SG. A significant decrease of *Dialister* was also observed at both two (fold decrease of 0.17; $p=1.6 \cdot 10^{-2}$) and six months (fold decrease of 0.3; $p=7.7 \cdot 10^{-3}$) post-SG. For *Corynebacterium* a weak significant increase was also observed at two (fold increase of 6.2; p=0.02) and six months (fold increase of 4.2; p=0.04) post-SG. *Oribacterium* was not present pre-SG, but showed a significant slight increase at two (relative abundance: $8.2 \cdot 10^{-5}$, p=0.02) and six months (relative abundance: $6.6 \cdot 10^{-5}$, p=0.04) post-SG. However, none of the changes in the relative abundance of the above-mentioned signature taxa remained statistically significant after correction for multiple testing.

To disentangle the short- and long-term effects of SG on the microbiota composition, we performed separate partial RDAs comparing the microbiota composition between: i). pre- and two months post-SG; ii) pre- and six months post-SG, and; iii) two and six months post-SG, respectively.

The short-term changes in the fecal microbiota composition, as indicated by the partial RDA on pre-SG and two months post-SG samples (p=0.004, figure 6.7A) were mainly characterized by an increase in the relative abundance of *Streptococcus, Rothia, Actinomyces, Atopobium, Mogibacterium, Oribacterium, Carynebacterium* and *Finegoldia,* and a decrease of *Dialister*. The RDA on the pre- and six months post-SG samples, demonstrated that the fecal microbiota composition was still significantly altered six months after surgery (p=0.008, figure 6.7b) and that these long-term alterations were still mainly driven by an increase in the relative abundances of *Streptococcus, Rothia* and *Actinomyces*. Other drivers for this separation were *Staphylococcus (*decreased in samples six months post-GS), *Akkermansia* and *Oribacterium* (both increased in six months post-SG samples, supplemental figure S6.2). A list of the most important taxa for the separation and their corresponding importance score can be found in the supplemental table 6.3 and 6.4. To detect which alterations might be related to the surgery itself or to the physiological changes induced by the gastric sleeve, we compared the behavior of the top 30 taxa from the RDA comparisons of T1 vs T2 versus T1 vs T3 (supplemental figure S6.3). These comparisons indicated that the relative abundance of *Staphylococcus* was increased two months post-SG, but decreased six months post-SG as compared to baseline (supplemental figure S6.2), suggesting that *Staphylococcus* is more likely to be temporarily affected by the surgical procedure, rather than the physiological changes due to the gastric sleeve. No other bacterial taxa behaved differently two months or six months post-SG. Moreover, in agreement with the observation that the most prominent shifts in bacterial taxa following SG were observed both two and six months after surgery, partial RDA did also not demonstrate separation of samples collected two and six months post-SG (P=0.47).

Figure 6.5. RDA based on the fecal microbiota composition of healthy individuals (circles) and obese patients pre-GS (squares).

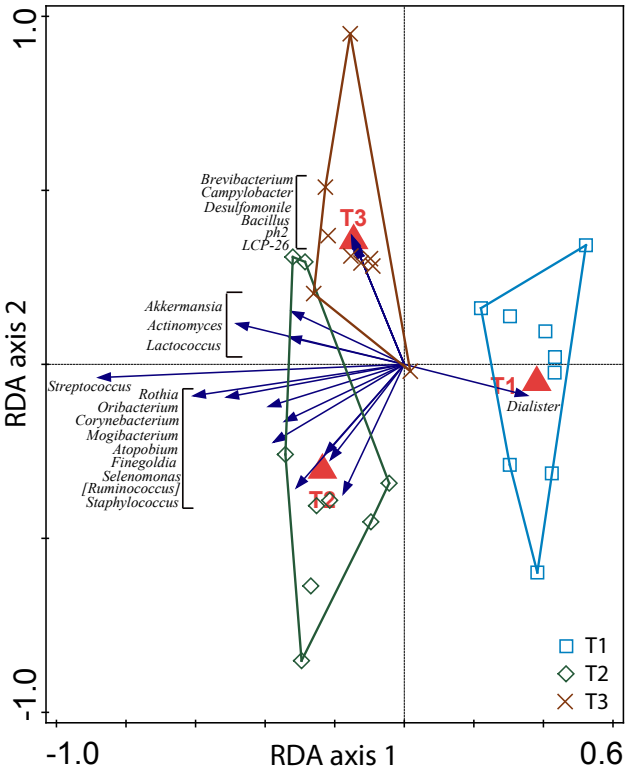


The 20 taxa that contributed most to the separation are represented by arrows. The effect of the variable BMI is represented by the red arrow.

Potential confounding effect on the microbiota

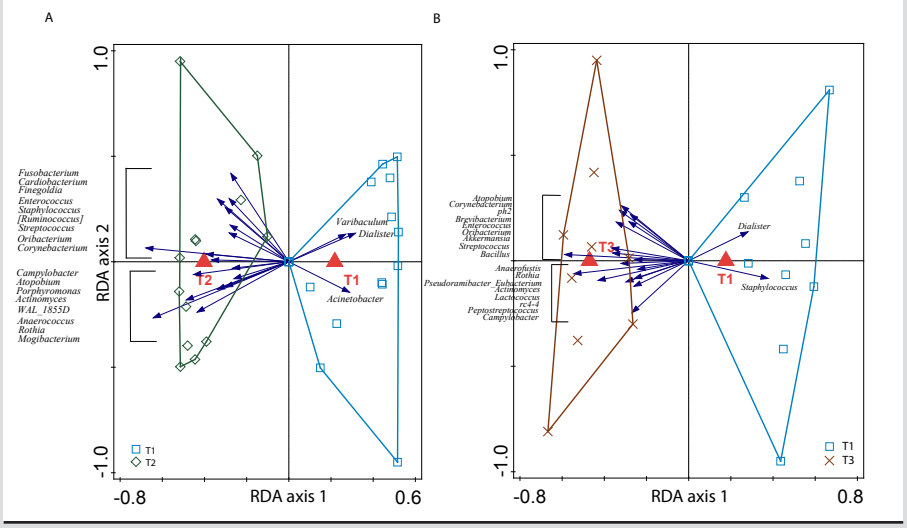
Neither within the lean subjects nor in the obese subjects at baseline, fecal protein concentration, diabetes, gender, smoking, alcohol and medication use were associated with the microbiota composition, indicating that potential confounding by these variables within our study was not likely (supplemental table 6.5).

Figure 6.6. RDA based on the fecal microbiota composition of obese individuals pre-GS (squares), two (diamonds) and six months (crosses) post-GS.



The 20 bacterial taxa that contributed most to the separation are represented by arrows.

Figure 6.7. RDA based on the fecal microbiota composition of obese individuals pre-GS (squares) and two months post-SG (diamonds) (A) and RDA based on the fecal microbiota composition of obese individuals pre-SG (squares) and six months post-SG (crosses) (B).



Bacterial taxa that contributed most to the separation are represented by arrows.

Associations between fecal microbiota composition and markers of intestinal permeability, inflammation, and glycemic control

A significant correlation was found between hsCRP and the overall fecal microbiota composition in obese patients prior to the SG procedure (p=0.002), but such a correlation was not found in the lean (p=0.632) nor in the obese individuals two (p=0.27) and six (p=0.26) months post-SG. For fecal calprotectin no such correlations were found. Furthermore, no correlation was found between fecal calprotectin, Hba1c or intestinal permeability markers and the microbiota composition of lean individuals or obese individuals pre- or post-SG (supplemental table 6.6).

Despite the absence of an association with the overall microbiota composition, a Pearson product-moment correlation analysis was performed in the obese group to examine whether changes in glycemic control (*i.e* Hba1c), inflammatory (hsCRP and fecal calprotectin) and intestinal permeability markers were associated with the microbial taxa that were most strongly affected by the SG (*i.e. Streptococcus, Rothia* and *Actinomyces*). The relative change in abundance of *Streptococcus* between baseline and two months post-SG was positively correlated with Hba1c, but did not withstand correction for multiple testing (p=0.01, FDR corrected 0.17). No further correlations were found between the bacterial taxa and permeability nor with inflammatory markers (supplemental table 6.7).

DISCUSSION

In the current study, we compared the fecal microbiota between morbidly obese and lean individuals and subsequently investigated the impact of sleeve gastrectomy on the morbidly obese fecal microbiota. Our results confirm that morbidly obese individuals have a microbiota composition that is distinct from lean individuals. The microbiota composition of morbidly obese subjects altered significantly following SG and these alterations persisted up to 6 months post-surgery. However, the post-SG microbiota did not change towards a lean profile, nor did the microbial alterations post-SG correlate with markers of inflammation, glycemic control or intestinal permeability.

Compared to lean individuals, the microbiota of morbidly obese individuals prior to SG was amongst others characterized by a higher F/B ratio, reduced microbial diversity and a lower abundance of *Bacteroides, Sutterella, Lachnospira* and *Butyricimonas*, which is in agreement with findings of several previous studies [8,26,41–43]. Moreover, a higher relative abundance of several SCFA-producing genera, including *Eubacterium, Dorea, Ruminococcus,* and *Blautia,* was observed in obese as compared to lean individuals. This is in line with multiple studies that reported higher fecal SCFA levels in obese individuals, being supportive for increased energy harvest as potential contributive factor [6,14,44–46]. BMI was found to be driving the separation of the microbiota of obese and lean individuals, whereas gender, age, smoking, metformin, antidepressants and PPI use, did not contribute to separation of microbiota profiles, confirming that the observed association between the microbiota composition and obesity was not caused by these confounding factors.

Overall, the microbiota structure at two and six months post-SG was profoundly changed as compared to pre-SG, but not towards the microbial profiles of lean subjects. Previous studies have shown that the alterations in the microbiota of obese subjects following RYGB are also not towards a lean, healthy microbiota composition [8,26], whereas dietary restriction was reported to change the microbiota of obese subjects towards a lean microbiota profile [47,48]. We checked for potential confounders, but the microbiota profiles pre- and post-SG did not correlate with fecal protein concentrations as marker of fecal consistency, gender, alcohol use, smoking and medication use, including metformin, a diabetic medication previously shown to have an effect on the microbiota [49]. However, it should be acknowledged that we cannot completely rule out an effect due to the low numbers of participants that used metformin in our study.

Although there were some bacterial taxa that were only altered at either two or six months post-SG, the majority of microbial changes were rather consistent at two and six months post-SG, supporting the notion that the microbiota changes are not merely a transient effect

of the surgical procedure or antibiotic prophylaxis, but more likely result from a combination of changes in the gastrointestinal physiology and diet. An increase of facultative anaerobic bacteria characteristic for the upper gastrointestinal tract, including *Streptococcus, Rothia* and *Actinomyces,* were amongst the most discriminative features of the microbiota post as compared to pre-SG. This may result from the decreased gastric acid production and accelerated gastric transit time reported after a SG procedure, which could promote the survival of upper GI microbes, resulting in an increase of these in the colon [25,50]. Damms-Machado *et al* mainly observed a reduction of butyrate-producing bacteria in five obese individuals three and six months post-SG, whereas these bacterial taxa were not found to be significantly affected in our study. These apparently disconcordant findings might (in part) be explained by the differences regarding dietary guidance between the two studies. Participants in the study by Damms-Machado *et al* followed a postoperative nutrition care program including a diet low in fiber and starch, which could explain the reduction in SCFA-producers.

Our results are partly in line with previous studies on the impact of RYGB on the microbiota composition which also reported an increase in facultative anaerobic bacterial taxa, although mainly belonging to members of the Proteobacteria [8,26–29,51,52].

The gastric sleeve procedure leaves the intestinal tract distal from the pylorus intact and therefore changes in the microbiota were, in contrast to microbiota alteration upon RYGB, hypothesized to be more likely associated with weight loss and less impacted by changes in gastrointestinal physiology. Remarkably, in the present study, as well as in previous studies on the microbiota following RYGB, the microbiota did not change towards a more lean profile six months after the bariatric procedure.

A correlation between the obese microbiota prior-SG with hsCRP was found, suggesting a relation specifically between the obesogenic microbiota composition and systemic inflammation. However, changes in biomarkers for systemic inflammation, intestinal permeability and glycemic control following SG, did not correlate with shifts in the microbiota composition. Furthermore, despite the weight loss and improvement in these biomarkers (*i.e.* hsCRP and Hba1c), the microbiota composition did not change towards a lean microbiota profile. It seems therefore unlikely that the microbiota is involved in the improvement seen in these biomarkers for obesity-related comorbidities. Microbial shifts should more likely be considered to be affected by the SG procedure and the subsequent changes in gastrointestinal physiology. These changes include a decreased production of gastric production and an increased gastric transit time, which could promote the survival of the oral microbiota. To what extend these microbial shifts might have on host health, needs to be investigated in future mechanistic studies. Although the morbidly obese individuals showed a significant weight loss, the median BMI six months post-SG (*i.e.* 34.8) still points towards an obese phenotype and a prolonged follow-up period

is necessary to investigate whether the microbiota changes induced by the SG procedure are persistent even after a BMI is achieved within the more physiological ranges.

The main strength of this study is the longitudinal study design enabling us to monitor the microbial changes upon sleeve gastrectomy within the same patients over time. Furthermore, by including an age and gender matched lean control group, we were able to compare baseline obese and SG-induced microbial alterations with the microbiota composition in lean subjects. In addition, we included a vast amount of clinical data that were available for analysis. Therefore, we were able to control for the effect of known potential confounders, such as diabetes, medication use, gender and smoking [53]. A limitation of our study is the lack of detailed information on dietary intake before and after the gastric sleeve procedure. Therefore, we cannot exclude that the observed microbial changes are (partially) affected by altered dietary intake. Assessment of the dietary intake is essential in future studies investigating the effect of bariatric surgery on the microbiota, considering the significant impact diet has on the microbiota composition [54]. In conclusion, we found a unique shift of the microbiota composition up to six months after the SG procedure (mainly driven by an increase of facultative anaerobic bacteria that are numerous in the upper gastrointestinal tract), which however did not change towards a more lean profile. The relevance of these changes for the host-physiology needs further study. Furthermore, more long-term observational as well as mechanistic studies (*e.g.* transplantation of the post-SG microbiota in germ-free animals) are warranted to investigate whether the changes of the microbiota upon bariatric surgery are persistence on the long-term and to what extend these changes impact the host physiology and metabolic improvements.

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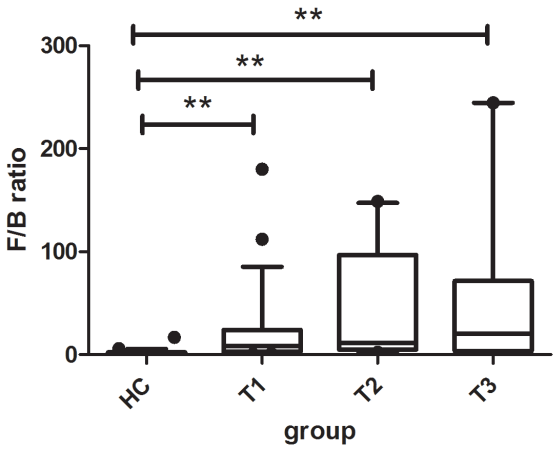
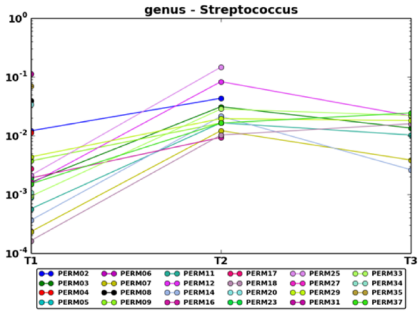
SUPPLEMENTAL INFORMATION

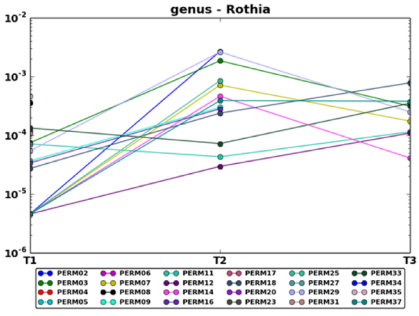
S 6.1. The fecal Firmicutes/Bacteroidetes (F/B) ratio in healthy individuals (HC), obese patients pre-SG (T1), obese patients two months (T2) and six months (T3) post-GS.

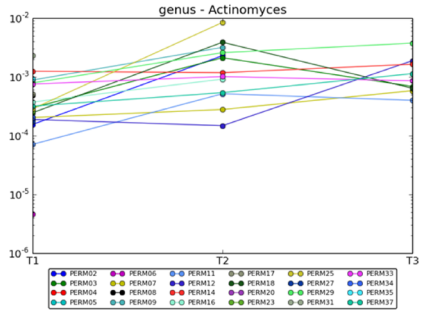


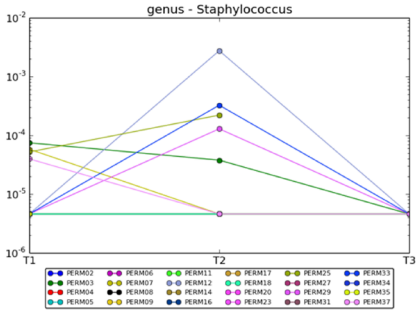
Data are presented as boxplots displaying medians with interquartile ranges. ** indicates p<0.0001.

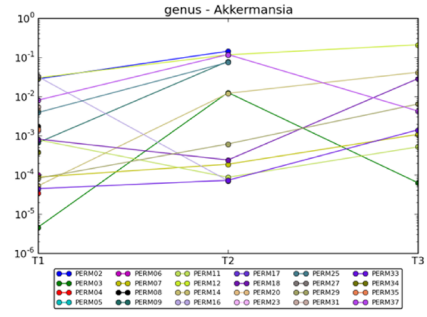
S6.2. Relative abundances of specific taxa at specified time points (pre-SG (T1), two (T2) and six (T3) months post-SG).

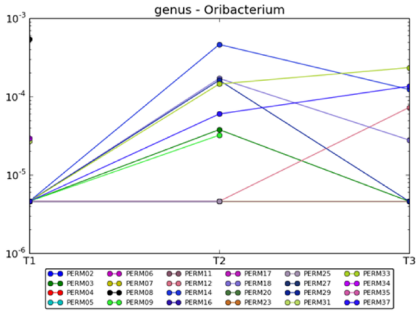


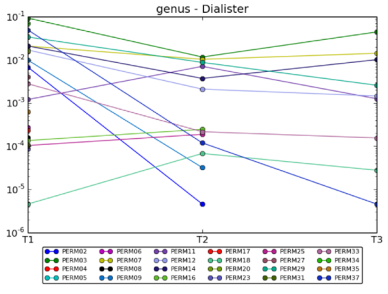




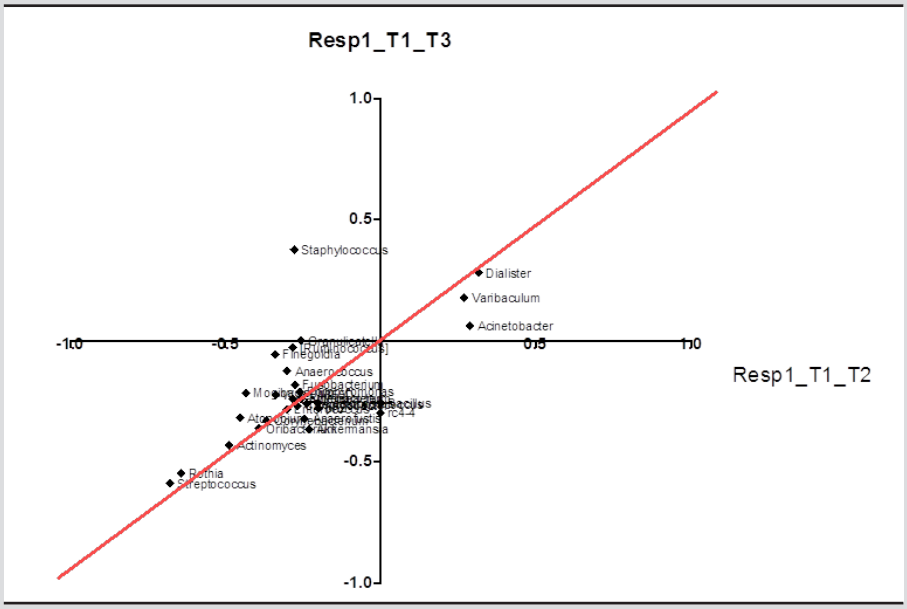








S6.3. Scatterplot based on the coordinates of the most important taxa according to the RDAs comparing samples T1 (pre-SG) versus T2 (2 months post-SG) and RDAs comparing samples T1 (pre-SG) versus T3 (6 months post-SG).



Supplemental table 6.1. Taxa that contributed most to the separation between healthy individuals (HC) and obese individuals pre-SG (T1) according to their importance scores.

Taxa important for HC vs T1	importance score
Odoribacter	0.7567
Bacteroides	0.7068
[Eubacterium]	0.6681
Sutterella	0.6581
Lachnospira	0.6467
Butyricimonas	0.6310
Actinomyces	0.6228
Dorea	0.6014
[Ruminococcus]	0.5914
Parabacteroides	0.5664
Blautia	0.5059
Peptococcus	0.3183
Anaerostipes	0.3045
Paraprevotella	0.2640
Bulleidia	0.2634
Mitsuokella	0.2115
Catenibacterium	0.1597
Desulfovibrio	0.1356
[Prevotella]	0.0412
Prevotella	0.0235

Supplemental table 6.2. Taxa that contributed most to the separation between obese individuals pre-SG (T1), two months (T2) and six months (T3) post-SG according to their importance scores.

Taxa important for T1 vs T2 vs T3	importance score
Streptococcus	0.8789
Rothia	0.6062
Oribacterium	0.5109
Actinomyces	0.4825
Corynebacterium	0.3900
Atopobium	0.3757
Dialister	0.3535
Mogibacterium	0.3439
Lactococcus	0.3305
Akkermansia	0.3239
[Ruminococcus]	0.3101
Finegoldia	0.2277
Selenomonas	0.2121
Staphylococcus	0.1754
Brevibacterium	0.1513
Campylobacter	0.1500
Bacillus	0.1467
Desulfomonile	0.1448
ph2	0.1426
LCP-26	0.1387

Supplemental table 6.3. Taxa that contributed most to the separation between obese individuals pre-SG (T1) and obese individuals two months (T2) post-SG.

Taxa important for T1 vs T2	importance score
Streptococcus	0.6780
Rothia	0.6427
Actinomyces	0.4866
Atopobium	0.4513
Mogibacterium	0.4347
Oribacterium	0.3923
Corynebacterium	0.3667
WAL_1855D	0.3384
Finegoldia	0.3382
Dialister	0.3156
Enterococcus	0.3013
Anaerococcus	0.3004
Acinetobacter	0.2875
[Ruminococcus]	0.2830
Cardiobacterium	0.2828
Staphylococcus	0.2786
Fusobacterium	0.2750
Varibaculum	0.2697
Campylobacter	0.2670
Porphyromonas	0.2604

Supplemental table 6.4. Taxa that contributed most to the separation between obese individuals pre-SG (T1) and obese individuals six months (T3) post-SG.

Taxa important for T1 vs T3	importance score
Streptococcus	0.5878
Rothia	0.5444
Actinomyces	0.4298
Staphylococcus	0.3768
Akkermansia	0.3652
Oribacterium	0.3610
Brevibacterium	0.3434
Corynebacterium	0.3286
Anaerofustis	0.3205
Atopobium	0.3178
rc4-4	0.2976
Enterococcus	0.2828
Dialister	0.2811
ph2	0.2760
Campylobacter	0,2683
Peptostreptococcus	0.2594
Lactococcus	0.2571
Bacillus	0.2554
Pseudoramibacter_Eubacterium	0.2412

Supplemental table 6.5. Confounder analysis of patient characteristics by means of RDA.

Variable tested	Study group	Explained variation (%)	P-value
Diabetes	T1	3.2	0.84
Metformin	T1	5.4	0.20
Metformin	T2	8.3	0.46
Metformin	T3	15.4	0.19
Gender	HC	2.9	0.78
Gender	T1	4.0	0.52
Smoking	T1	8.3	0.57
Antidepressants	T1	6.0	0.98
PPI	T1	4.1	0.54

Supplemental table 6.6. Associations between host markers and the microbiota composition as examined by CCA.

Variable tested	Study group	Explained variation (%)	P-value
Calprotectin	HC	6.2	0.05
Calprotectin	T1	4.8	0.29
Calprotectin	T2	6.9	0.84
Calprotectin	T3	15.0	0.17
hsCRP	HC	3.0	0.63
hsCRP	T1	9.7	0.002
hsCRP	T2	9.2	0.27
hsCRP	T3	13.9	0.26
L/R ratio	HC	3.6	0.56
L/R ratio	T1	4.2	0.47
L/R ratio	T2	9.4	0.40
L/R ratio	T3	15.5	0.10
protein content	HC	4.2	0.31
protein content	T1	4.6	0.37
protein content	T2	11.2	0.24
protein content	T3	14.7	0.26
Hba1c	T1	4.9	0.62
Hba1c	T2	11.7	0.22
Hba1c	T3	18.0	0.07
S/E ratio	HC	1.4	0.31
S/E ratio	T1	3.3	0.89
S/E ratio	T2	6.2	0.92
S/E ratio	T3	13.1	0.36
sucrose	HC	3.7	0.51
sucrose	T1	4.5	0.48
sucrose	T2	9.9	0.17
sucrose	T3	11.5	0.68

Supplemental table 6.7. Pearson correlation coefficients (R) between changes in the three most strongly affected taxa by SG and markers of glycemic control, inflammatory and intestinal permeability.

	Streptococcus			Actinomytes			Rothia		
Variable	R	p	FDR	R	p	FDR	R	p	FDR
hba1c ΔT1-ΔT2	0.796	0.010	0.225	-0.154	0.693	0.846	-0.560	0.117	0.642
hba1c ΔT1-ΔT3	0.526	0.180	0.692	-0.520	0.186	0.692	0.307	0.460	0.764
CRP ΔT1-ΔT2	0,314	0.300	0.705	0.021	0.946	0.968	-0.152	0.620	0.801
CRPc ΔT1-ΔT3	0.384	0.308	0.713	-0.532	0.141	0.687	0.474	0.198	0.692
calprotectin ΔT1-ΔT2	0.176	0.565	0.801	0.240	0.429	0.738	0.409	0.166	0.692
calprotectin ΔT1-ΔT3	0.417	0.264	0.692	-0.429	0.249	0.692	0.654	0.056	0.434
sucrose ΔT1-ΔT2	0.158	0.625	0.720	-0.343	0.276	0.434	-0.584	0.046	0.738
sucrose ΔT1-ΔT3	0.629	0.070	0.801	-0.669	0.049	0.692	0.303	0.428	0.434
SE ΔT1-ΔT2	0.183	0.549	0.801	0.091	0.767	0.855	-0.112	0.717	0.852
SE ΔT1-ΔT3	0.007	0.986	0.986	0.298	0.436	0.738	-0.109	0.779	0.855
LR ΔT1-ΔT2	0.285	0.369	0.738	0.354	0.260	0.692	0.161	0.617	0,801
LR ΔT1-ΔT3	-0.318	0.405	0.738	0.236	0.541	0.801	-0.112	0.774	0.855
BMI ΔT1-ΔT2	0.376	0.229	0.801	0.161	0.617	0.924	-0.297	0.349	0.801
BMI ΔT1-ΔT3	0.214	0.580	0.692	0.069	0.861	0.801	-0.220	0.569	0.732

7

Altered fecal and duodenal microbial profiles are associated with disease severity in liver cirrhosis patients

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Submitted

ABSTRACT

Background & Aims: The relevance of monitoring fecal and mucosal microbial perturbations in liver cirrhosis patients in clinical practice remains to be determined. The 16S-23S interspacer region-based profiling (IS-pro) has potential to rapidly assess and monitor intestinal microbiota. Therefore, we aimed to investigate the fecal and mucosa-associated microbiota in a heterogeneous group of cirrhosis patients in relation to disease-related factors.

Methods: Feces, sigmoid and duodenum biopsies of healthy controls (n=26, n=22 and n=18, respectively), compensated (n=25, n=12 and n=13, respectively) and feces of decompensated cirrhotic patients (n=16) were available for analysis of the microbiota composition by IS-pro.

Results: In feces, higher richness (median(range)) of Firmicutes and lower richness of Bacteroidetes was found in compensated (5.0(0.9-12.0) and 20.0(4.0-39.0), resp.) and decompensated cirrhotic ((6.5(1.0-22.0) and (18.5(0.0-26.0) resp.) versus controls (3.0(0.0-10.0) and (23.5(13-32) resp., all $P<0.05$). In duodenum, only an increased richness for Firmicutes was found in compensated cirrhotics (10.0(3.0-13.0)) versus controls (5.0(0.0-14.0), $P=0.01$), and a positive correlation between *Lactobacillus johnsonii* and lactulose/rhamnose ratio indicating small intestinal permeability. No significant microbial differences were found in sigmoid. Constrained ordination showed distinct fecal clusters based on disease severity by Child-Pugh classification, but not by demographics, etiology or medication.

Conclusions: Differences in fecal and duodenal, but not sigmoid microbiota were found between cirrhotics and controls. Alterations in the fecal microbiota were more pronounced with liver cirrhosis progression. Distinct clusters based on Child-Pugh classification, support the potential of IS-profiling as disease progression marker. The duodenal microbiota might be involved in the increased permeability of the small intestine.

INTRODUCTION

The intestinal microbiota is a complex ecosystem dominated by two phyla, *i.e.* Firmicutes and Bacteriodetes, whereas at lower taxonomic levels the microbiota consists of more than 1000 different species [1] . Increasing evidence indicates that the intestinal microbiota is involved in a variety of intestinal, systemic and liver diseases [2]. Microbial perturbations have been reported in patients with chronic liver diseases, such as non-alcoholic fatty liver disease (NAFLD) [3] and alcoholic liver disease (ALD) [4], which can progress to liver cirrhosis. This end-stage of chronic liver diseases can be divided into an asymptomatic (compensated) and symptomatic (decompensated) stage. The latter is defined by clinically evident complications, *i.e.* ascites, variceal hemorrhage, hepatic encephalopathy and/or jaundice [5]. Further insight in hepatic and extra-hepatic mechanisms contributing to disease progression, such as alterations in the intestinal microbiota and permeability, as well as non-invasive markers thereof, are relevant for early diagnosis and to identify targets for interventions.

In recent years, several studies characterized the structure and function of the fecal microbiota in patients with compensated and/or decompensated cirrhosis versus healthy controls. An increase of Proteobacteria and Fusobacteria, and a decrease of Bacteroidetes in mixed groups of (de)compensated patients versus healthy controls has been observed [6–8]. At the family level, *Enterobacteriaceae* [7–9], *Streptococcaceae* [7,8,10], and *Veillonellaceae* [7,8,10] were found to be increased, whereas *Ruminococcaceae* [9] and *Lachnosperiaceae* [8,9] were decreased in cirrhotic patients. The alterations seem to be more pronounced with advanced cirrhosis, especially in patients with complications, such as hepatic encephalopathy [11,12].

Most of the studies have been performed using state-of-the-art 16S rRNA gene sequencing, but this method is not readily applicable in clinical practice for on-demand analyses. The validated 16S-23S interspacer (IS) region-based profiling (IS-pro) may have potential as clinical tool to assess and monitor the microbiota structure, as it enables relatively fast analysis of individual samples [13]. Furthermore, abovementioned studies have focused primarily on cirrhotic patients with alcohol and/or viral infections as main cause. Data on cirrhotics with mixed etiologies and on mucosa-associated microbiota are limited. Fecal perturbations indicate a role of the large intestine, while a high prevalence of small intestinal bacterial overgrowth (SIBO) with increased risk of bacterial translocation, also warrants evaluation of the small intestine in liver cirrhosis [14].

Therefore, we aimed to investigate differences in both the fecal and mucosa-associated microbiota in a heterogeneous group of patients with liver cirrhosis versus controls by IS-profiling, taking into account disease-related factors. We hypothesized that the fecal, sigmoid and duodenal microbiota composition differs in liver cirrhosis patients versus healthy controls and is affected by disease progression.

MATERIALS AND METHODS

Patients and study design

Patients with stable compensated liver cirrhosis of various etiology (*i.e.* without clinically evident complications, including ascites, variceal hemorrhage, hepatic encephalopathy and/or jaundice) and age-, sex and BMI-matched healthy volunteers participating in a prior case-control study on intestinal permeability, were available for analyses of the intestinal microbiota [15]. In addition to biological samples, demographic and clinical characteristics (*i.e.* severity, etiology and drug therapy) were available, as well as urinary sugar excretions as indicators of small (lactulose/rhamnose (L/R) ratio) and large intestinal permeability (sucralose/erythritol (S/E) ratio) [15].
Briefly, 26 compensated liver cirrhosis (CLC) patients and 27 healthy controls (HC) collected a fresh fecal sample. Aliquots were stored within 12 hours after defecation at -80ºC until further analysis. A subset of patients (*n*=15) and controls (*n*=22) underwent a gastroduodenoscopy and/or sigmoidoscopy after an overnight fast without prior bowel cleansing. Biopsies were obtained from the second segment of the duodenum and the sigmoid approximately 20 cm from the anal sphincter. Biopsies were snap-frozen in liquid nitrogen and stored at -80°C until further processing.

In addition, fresh frozen fecal samples were available from inpatients with decompensated liver cirrhosis (DLC) (*n*=16), as defined by the presence of one or more of the following clinically evident complications: ascites, variceal hemorrhage, hepatic encephalopathy and/or jaundice. Severity of liver cirrhosis of CLC and DLC was scored by the Child-Pugh classification [16].

The study has been approved by the Medical Ethics Committee of Maastricht University Medical Center (MUMC), was conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul) and registered at the US National Library of Medicine (http://www.clinicaltrials.gov, NTC01081236). All subjects gave written informed consent prior to participation.

DNA isolation and 16S-23S IS profiling of the intestinal microbiota

Metagenomic bacterial DNA was isolated from fecal and biopsy samples with the NucliSENS® easyMag® automated DNA isolation machine (Biomérieux, Marcy l'Etoile, France). Amplification of the 16S-23S rRNA IS-regions was performed with the IS-pro assay (IS-Diagnostics, Amsterdam, the Netherlands). DNA fragment analysis was performed on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). Further information can be found in the supplement.

Data analyses

Subject characteristics are presented as median (range) and were compared between groups using the Mann-Whitney *U*-test for non-parametric data for continuous variables and the χ^2 test for dichotomous variables. A *P*-value<0.05 was considered statically significant using a

two-tailed test.
IS-pro data consist of peaks with a specific length and its height (relative fluorescence units (RFU)), corresponding to the abundance. All RFUs were log2 transformed for downstream analysis to reduce dominance of high peaks and to include less abundant species. A heatmap was made based on all IS-profiles using Spotfire (TIBCO, Palo Alto, USA). The observed peaks, Shannon index and Bray-Curtis (beta-diversity) dissimilarity were calculated as described previously [17].

The Mann-Whitney *U*-test was used to analyze differences in Firmicutes/Bacteroidetes (F/B) ratio, richness and the Shannon diversity index between CLC, DLC and HC, using SPSS version 20.0. To test for differences in peak frequency between HC and CLC, the Kruskal-Wallis test was applied, using a false discovery rate (FDR) of q-value of ≤ 0.15 to correct for multiple testing. A Pearson product-moment correlation analysis was performed to investigate the relationship between sugar ratios (S/E and L/R) and peaks with a significantly different frequency between HC and CLC according to the Kruskal-Wallis test.

The microbiota structure between groups was compared using the nonparametric t-test (using Monte Carlo permutations to calculate significances in Qiime 1.9) and visualized by Principle coordinate analysis (PCoA) plots of Bray-Curtis dissimilarities. To analyze the effect of disease status, sex, age, BMI or smoking on the microbiota community structure, a distance-based redundancy analysis (db-RDA) was performed on the Bray-Curtis distance matrix using the capscale function in R. Subsequently, an ANOVA-like permutation test was applied to determine statistical significance of individual variables. An additional db-RDA was performed on the fecal and mucosal samples of CLC, to investigate whether disease severity, etiology and drug therapy did affect the overall microbiota structure.

RESULTS

Patients

Fecal samples were obtained from 27 HC, 26 CLC and 16 DLC patients. Subject characteristics are given in table 7.1. Serum alanine transaminase (ALT) and gamma-glutamyl transferase (GGT) were significantly increased in CLC versus HC (ALT; *P*=0.025, GGT; *P*<0.001). Furthermore, 17 and 9 CLC were classified as Child-Pugh class A and B, respectively. DLC patients had significantly higher serum ALT and GGT levels than HC (ALT; *P*=0.002, GGT; *P*<0.001), and higher GGT versus CLC (*P*=0.042). The Child-Pugh was also significantly higher in DLC versus CLC (*P*<0.001).

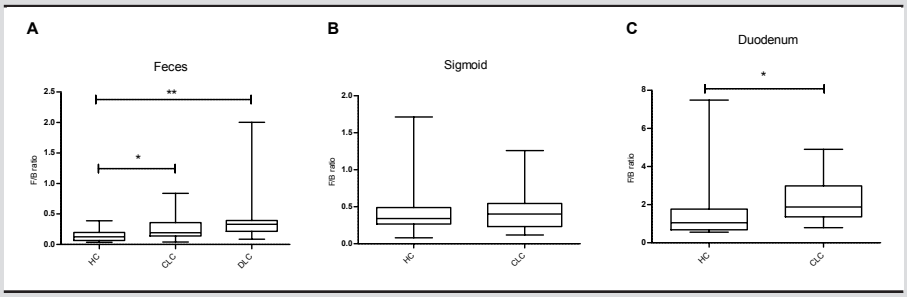
IS-pro data

F/B ratios were significantly higher in fecal samples (*P*=0.004, Figure 7.1A) and duodenal biopsies (*P*=0.039, Figure 7.1C) of CLC versus HC, while no differences were observed in sigmoid mucosa (*P*>0.05, Figure 7.1B). The F/B ratio in fecal samples was also significantly higher in DLC versus HC (P<0.001), but not when compared to CLC (Figure 7.1A).

Microbial richness and diversity in faeces and mucosal biopsies

In fecal samples, total bacterial richness and diversity were similar between CLC and HC, as indicated by the number of observed peaks (Figure 7.2) and the Shannon index, respectively. However, richness and diversity of Firmicutes were significantly higher in CLC than HC (*P*=0.03 and *P*=0.02 respectively), while richness, but not diversity of Bacteroidetes was significantly lower in CLC versus HC (*P*=0.04). The observed differences were even more pronounced in DLC (Figure 7.2). No differences were found with regard to the Proteobacteria.

Figure 7.1. Firmicutes/Bacteroidetes ratios of healthy controls (HC), patients with compensated cirrhosis (CLC) and patients with decompensated cirrhosis (DLC).



A: Firmicutes/Bacteroidetes ratio in fecal samples. B: Firmicutes/Bacteroidetes ratio in sigmoid biopsies. C: Firmicutes/Bacteroidetes ratio in duodenal biopsies. Data are presented as boxplots displaying median with range (*P < 0.05, **P < 0.01).

Table 7.1. Characteristics of subjects

	Healthy controls (*n* = 27)	Patients with compensated cirrhosis (*n* = 26)	Patients with decompensated cirrhosis (*n* = 16)
Age (years)	60 (19-78)	61 (18-72)	59 (39-81)
Sex (M / F)	17 / 10	17 / 9	12 / 4
BMI (kg/m²)	26.0 (18.1-32.1)	25.8 (18.8-39.0)	26.7 (20.1-40.0)
Current smokers (%)	3 (11.1)	10* (38.5)	8* (50.0)
ALT (U/L)	22.0 (10.0-32.0)	28.0 (9.0-130.0)*	35.0 (14.0-137.0)*
GGT (U/L)	22.0 (6.0-50.0)	51.0 (17.0-259.0)* **	80.0 (29.0-1890.0)*
Etiology (%):			
- alcohol-related		11 (42.3)	12 (75.0)
- autoimmune-related		6 (23.1)	1 (6.3)
- metabolic		2 (7.7)	2 (12.5)
- chronic viral infection		1 (3.8)	1 (6.3)
- cryptogenic		5 (19.2)	0 (0.0)
- multifactorial		1 (3.8)	0 (0.0)
Child Pugh class (A/B/C)	-	17/ 9/ 0	0 / 7/ 9
Child-Pugh score[a]	-	5.0 (5.0-9.0)**	10.0 (7.0-13.0)
MELD-score	-	8.0 (6.0-15.0)**	13.0 (7.0-22.0)
Drug therapy (%)			
- antibiotics		1 (3.8)	10 (62.5)
- pre/probiotics		4 (15.4)	2 (12.5)
- laxatives		7 (26.9)	7 (43.8)
- proton pump inhibitors		15 (57.7)	9 (56.3)
- ursodeoxycholic acid		11 (42.3)	2 (12.5)
- glucocorticosteroids/ immunosuppressives		4 (15.4)	1 (6.3)
Liver biopsy (%)	-	16 (61.5)	5 (31.3)
S/E ratio in 5-24 h urine	(0.008-0.034)	0.019 (0.008-0.051)*	-
L/R ratio in -5 h urine	0.023 (0.006-0.069)	0.023 (0.010-0.115)	

**P*<0.05 versus healthy controls

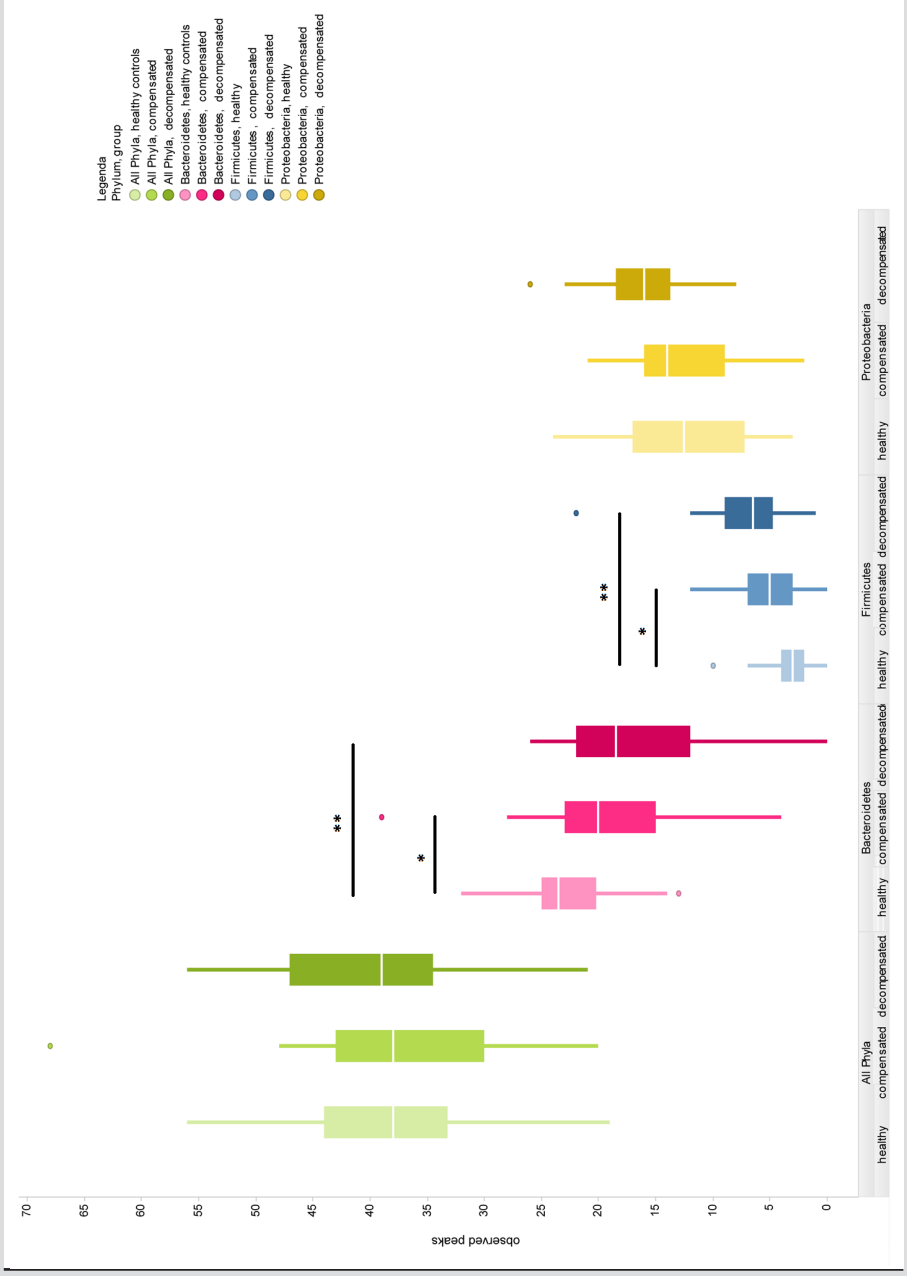
***P*<0.05 versus patients with decompensated cirrhosis

[a] One patient with compensated cirrhosis had a Child-Pugh score of 9, due to high bilirubin levels because of Gilbert's Syndrome.

L/R = lactulose/rhamnose ratio

S/E = sucralose/erythritol ratio

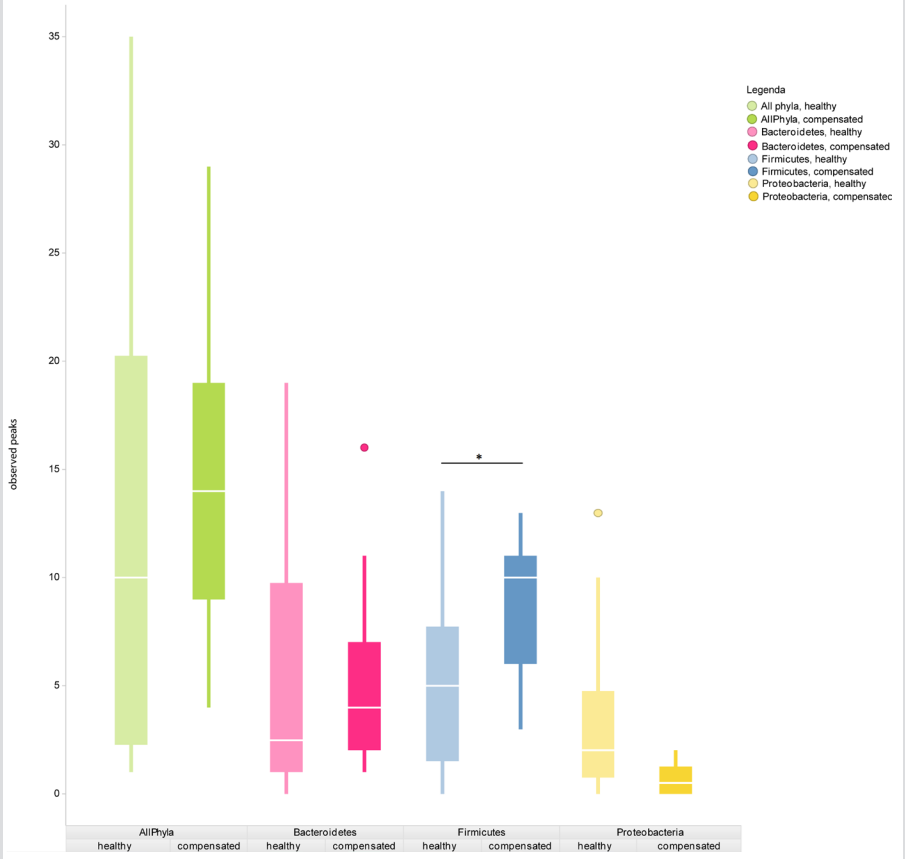
Figure 7.2. Species richness (number of observed peaks) in fecal samples of healthy controls, patients with compensated cirrhosis and patients with decompensated cirrhosis of all phyla combined (green), Bacteroidetes (pink), Firmicutes (blue) and Proteobacteria (yellow).



Data are presented as boxplots displaying the interquartile range with median and outliers (*P < 0.05, **P < 0.01).

Duodenal and sigmoid biopsies were available from CLC and HC. Bacterial richness and diversity of sigmoid biopsies did not differ between CLC and HC (data not shown). In duodenal biopsies however, Firmicutes richness was significantly increased in CLC versus HC (P=0.01, Figure 7.3), whereas no differences were found for diversity, nor for richness and diversity of the total bacterial profiles, Bacteroidetes or Proteobacteria.

Figure 7.3. Species richness (number of observed peaks) in duodenal biopsies of healthy controls and patients with compensated cirrhosis of all phyla combined (green), Bacteroidetes (blue), Firmicutes (pink) and Proteobacteria (yellow).



Data are presented as boxplots displaying the interquartile range with median and outliers (*P < 0.05, **P < 0.01).

Fecal microbial community structure

A heat map was generated including the fecal IS-profiles of all subjects. Hierarchical clustering indicated that the fecal microbiota composition of patients with cirrhosis and HC were

different (supplemental figure S7.1). PCoA comparisons of Bray-Curtis dissimilarities showed stronger clustering of HC than of CLC (supplemental figure S7.2). The median (range) within-group Bray-Curtis dissimilarity was significantly larger for CLC (0.58 (0.41-0.79)) than for HC (0.53 (0.38-0.75)), indicating inter-individual variations in microbiota composition of HC were significantly smaller than those within CLC (*P*=0.01).

db-RDA of the Bray-Curtis dissimilarity demonstrated that the fecal samples did separate by health status (*i.e.* CLC vs HC, *P*=0.001) (Figure 7.4A), but not by age (*P*=0.216), sex (*P*=0.412), BMI (*P*=0.052), smoking (*P*=0.084) or alcohol use (*P*=0.667). Additionally, a significant effect was found for Child-Pugh class (*P*=0.010), but not MELD-score (*P*=0.312), ALT (*P*=0.066), GGT (*P*=0.461), etiology (*P*=0.756) and drug therapy, *i.e.* PPIs (*P*=0.395) and laxatives (*P*=0.222) for the CLC group (Figure 7.4D).

Although hierarchical clustering showed no clear separation between CLC and DLC, db-RDA showed distinct clusters based on disease severity [HC, patients with Child-Pugh A, B and C cirrhosis (*P*=0.001)]. Fecal samples of cirrhotic patients were more distant to HC when categorized in a more severe class (Figure 7.5).

Mucosal microbial community structure

PCoA of sigmoid biopsies did not show clustering based on health status (data not shown). Also no differences were found when comparing within-group Bray-Curtis dissimilarity between CLC (0.62 (0.46-0.86)) and HC (0.58 (0.38-0.96)). db-RDA of the Bray-Curtis dissimilarity showed no separation of sigmoid biopsies by health status (*P*=0.076), age (*P*=0.384), sex (*P*=0.380), BMI (*P*=0.608), smoking (*P*=0.093) or alcohol use (*P*=0.130) (Figure 7.4B). Additional db-RDA also showed no effect of disease severity (*P*=0.709) and drug therapy (*P*=0.260 for PPIs, *P*=0.710 for ursochol) (Figure 7.4E).

Duodenal biopsies of CLC seemed to cluster together according to the PCoA of Bray-Curtis dissimilarities (supplemental Figure S7.3). The within-group Bray-Curtis dissimilarity was significantly higher for HC (0.72 (0.36-0.95)) than for CLC (0.56 (0.38-0.79)), indicating smaller inter-individual variations for CLC as compared to HC (*P*=0.01). db-RDA demonstrated that the duodenal biopsies were separated by health status (*P*=0.04) (Figure 7.4C), but not by age (*P*=0.713), sex (*P*=0.051), BMI (*P*=0.512), smoking (*P*=0.755) or alcohol use (*P*=0.127). Also no significant effect of disease severity (*P*=0.135), etiology (*P*=0.619) or drug therapy (*P*=0.300 for PPIs, *P*=0.211 for laxatives) was found (Figure 7.4F)

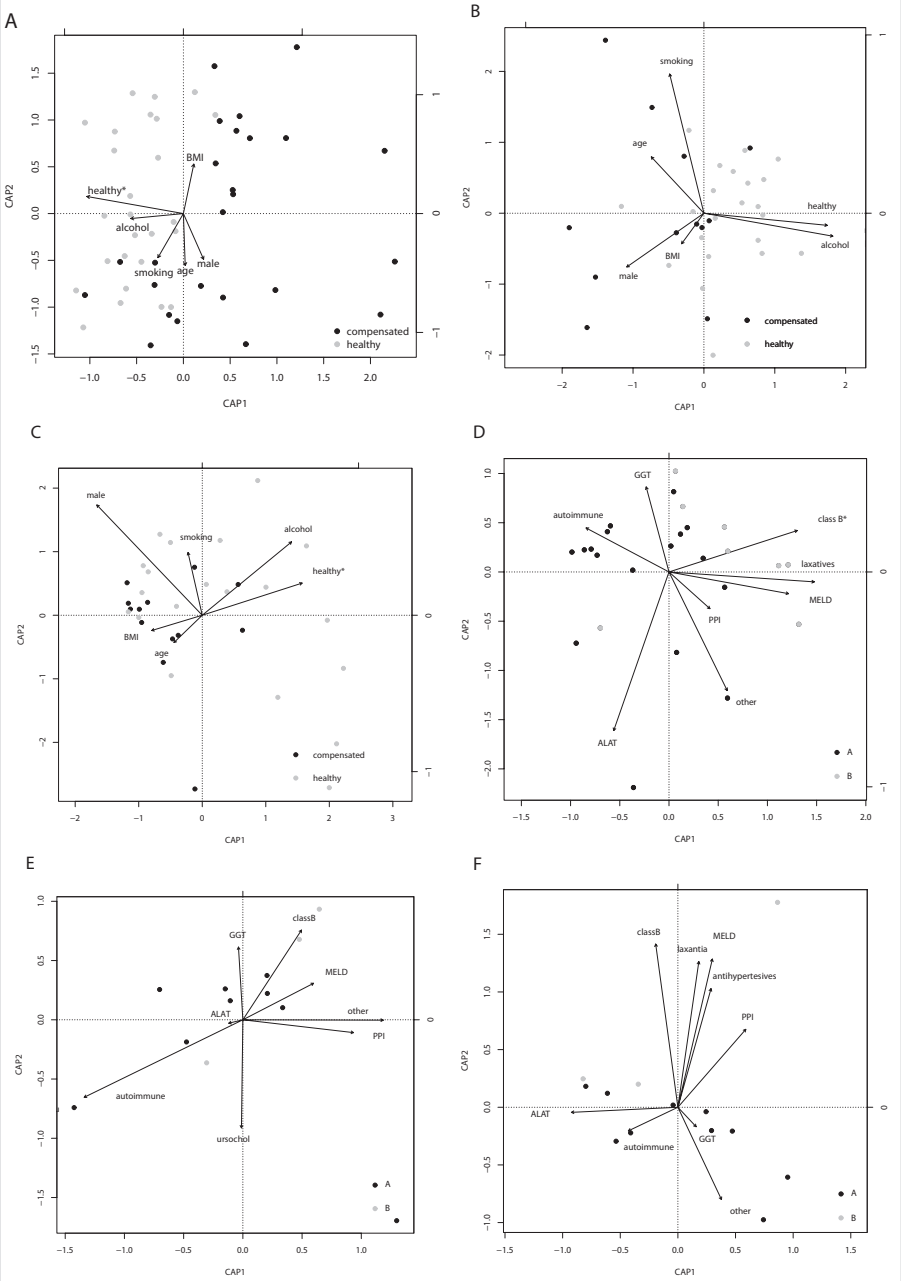
Bacterial taxa associated with compensated liver cirrhosis

The abundance of six and 12 peaks were found to be significantly different (FDR≤0.15) between CLC and HC in fecal and duodenal samples, respectively (Supplemental table 7.1). An increase

of *Streptococcus bovis/gallolyticus* and *Streptococcus mitis* (corresponding to four peaks for which identification was possible) was found in fecal samples of CLC versus HC. *Enterococcus* spp. *Staphylococcus* spp., *Lactobacillus johnsonii* and *Lactobacillus plantarum* (corresponding to nine peaks for which identification was possible) showed higher abundance in duodenal biopsies of CLC versus HC.

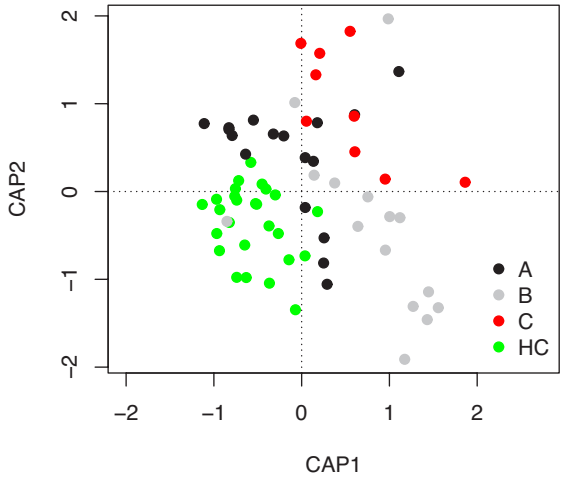
The fecal and duodenal peaks that significantly differed between CLC and HC were related to sugar ratios indicating large and small intestinal permeability, respectively. A significant positive correlation was found between the L/R ratio and the duodenal peaks 264_Firmicutes (P=0.03, r=0.43) and 265_Firmicutes (P=0.02, r=0.51), which both correspond to *L. johnsonii*. No significant correlation was found between the S/E ratio and differential peaks in fecal samples.

Figure 7.4. Distance based redundancy analysis (cd-DRA) ordination on Bray-Curtis dissimilarity of fecal (a, d), sigmoid (b, e) and duodenum (c, f) from patients with compensated cirrhosis (CLC) and samples from healthy control (a, b and c) or patients with compensated cirrhosis only (d, e and f).



The vectors indicate the the effect of each variable on the microbiota composition (*p<0.05)

Figure 7.5. Distance based redundancy analysis (dbRDA) ordination based on Bray-Curtis dissimilarity of samples from patients with healthy subjects, compensated and decompensated liver cirrhosis patients based on disease severity (Child-Pugh score).



DISCUSSION

The present study showed that both fecal and duodenal microbiota composition of patients with compensated liver cirrhosis of various etiologies differed from healthy controls, as shown by an increased fecal as well as duodenal F/B ratio in these patients, and alterations in richness and diversity of Firmicutes and Bacteroidetes. These differences were even more pronounced with progression of liver cirrhosis, *i.e.* in patients with decompensated cirrhosis. Furthermore, patients with (de)compensated cirrhosis were found to cluster separately from HC, and showed distinct clusters based on Child-Pugh classification. No alterations were found in the sigmoid microbiota composition.

In the present study, we included a heterogeneous group of patients with compensated and decompensated cirrhosis, and compared them with age-, sex-, and BMI-matched controls. In addition, duodenal and sigmoid biopsies were available for subgroups. The microbiota composition was assessed by IS-pro, which is easy to implement in clinical practice and enables analysis of individual samples with a turn-around-time from sample delivery to ready-to-interpret results of less in 5 hours.

Significant differences were found in the fecal microbiota composition of compensated cirrhotic patients compared to HC, resulting in an increased F/B ratio in the patients, which is in line with others [6–8]. Total microbial richness and diversity in fecal samples was not affected, whereas both richness and diversity of Firmicutes were significantly higher and richness of Bacteroidetes was significantly lower in the patients compared to controls. Furthermore, we found clustering of the subjects by health status on PCoA, and showed that the fecal microbiota composition of HC was more homogenous than that of compensated patients. These findings indicate that cirrhotic patients show a larger within-group variation, but overall have a different fecal microbiota profile than HC.

The observed microbial variation within the group of compensated patients may be associated with host characteristics or etiological factors. However, we did not observe any effect of alcohol use, etiology, BMI or drug therapy, but it should be noted that subgroups were rather small. Since only one compensated patient used antibiotics within 2 months prior to inclusion, this is unlikely to affect our findings. The observed microbial changes seem to be associated with compensated cirrhosis itself, for example by alterations in bile acid synthesis [18–20]. Interestingly, the severity of the disease (by Child-Pugh class) was found to be associated with the microbiota composition, with more pronounced differences in Child-Pugh B versus A patients when compared to controls. This effect was further supported by findings that the observed differences were more pronounced with disease progression, and are in line with findings by others [7,21]. It should however be noted that the perturbations in the decompensated group

might (at least in part) be affected by the high percentage antibiotic users in this group (63%).

Four out of six peaks of which the abundance in fecal samples differed significantly between controls and compensated cirrhotic patients, corresponded to known bacterial taxa. Most of them corresponded to *Streptococcus* spp., which include oral commensals [22]. Oral bacteria were also found by others to be increased in fecal samples of cirrhotic patients [6,10]. This may (in part) be caused by reduced bile acid synthesis, portal hypertension and/or altered motility [23–25].

As data on the mucosa-associated microbiota in patients with cirrhosis are limited and SIBO is frequently found in liver cirrhosis patients we also characterized the microbiota in sigmoid and duodenal biopsies in subgroups of compensated cirrhotics and controls [14].

In contrast to Bajaj *et al*, no alterations were observed in the microbiota composition of the sigmoid between compensated cirrhotics and controls [11]. In duodenal biopsies, the F/B ratio was significantly increased in compensated patients versus HC. Remarkably, these findings were in line with those of the fecal microbiota. Furthermore, microbial richness of Firmicutes was found to be significantly higher in duodenum of compensated cirrhotic patients than HC. Interestingly, the alterations resulted in a more homogeneous duodenal microbiota composition in the compensated patient group, and were not affected by demographics, disease severity, etiology nor drug therapy. These findings suggest that the compositional changes in the duodenum were most likely due to the cirrhosis and may point to involvement of the small intestine in its etiology.

Data from our group and from previous studies indicate that permeability of the small and/or large intestine is increased in cirrhotic patients [15,26]. Changes in the intestinal microbiota together with increased permeability, may contribute to bacterial translocation and disease progression. A significant positive correlation was found between *L. johnsonii* and the L/R ratio, indicating small intestinal permeability. No correlations were found with the S/E ratios (colonic permeability). The role of *L. johnsonii* in small intestinal permeability remains elusive as findings on its effects on barrier function are inconsistent [27–29]. To our knowledge, this is the first study that profiled the duodenal microbial composition in patients with cirrhosis, and therefore the significance of these findings should be further investigated.

In conclusion, this study showed the potential of IS-profiling to assess microbial changes in line with sequencing data. The observed gradient between Child-Pugh A, B and C cirrhosis shows that this technique has potential as marker to monitor disease progression. Furthermore, we found indications for involvement of the duodenal microbiota in liver cirrhosis and possible associations with small intestinal permeability, which warrant further evaluation.

CONFLICTS OF INTEREST

A.E.B. and P.H.M.S. have proprietary rights to the IS-pro technique and are co-owners of the spin-off company IS-Diagnostics.

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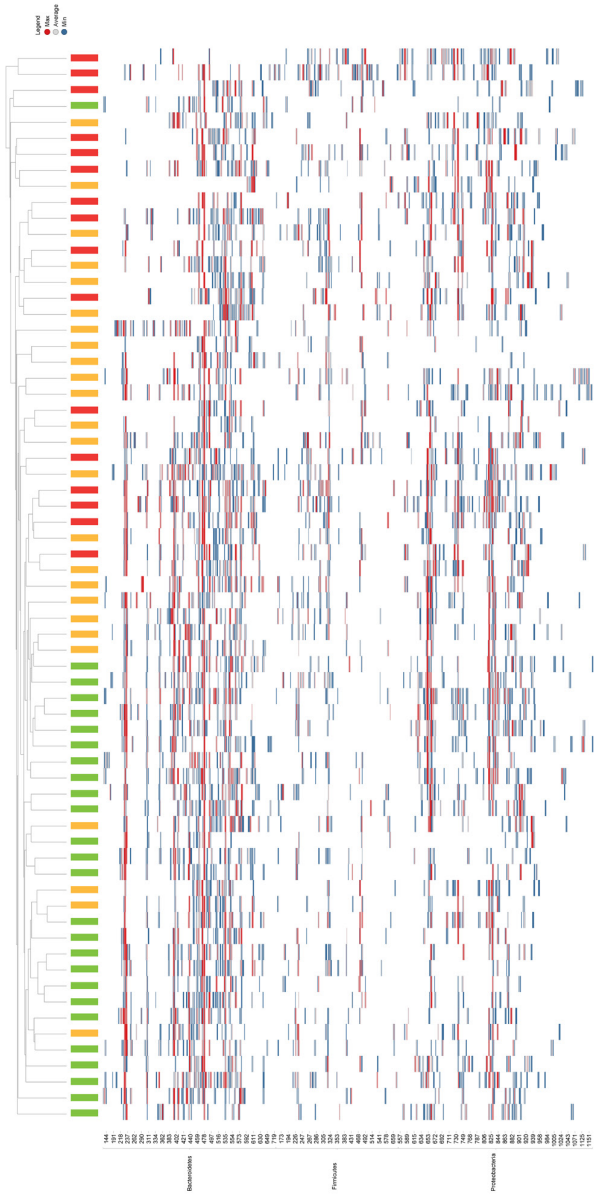
SUPPLEMENTAL INFORMATION

S7.1. Heat map of IS-profiles from all fecal samples of healthy controls (green) and patients with compensated and decompensated cirrhosis (red).

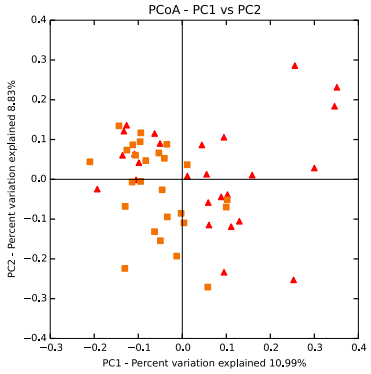


Rows are sorted on phylum level and clustering was performed by Unpaired Pair Group Method with arithmetic Mean (UPGMA)

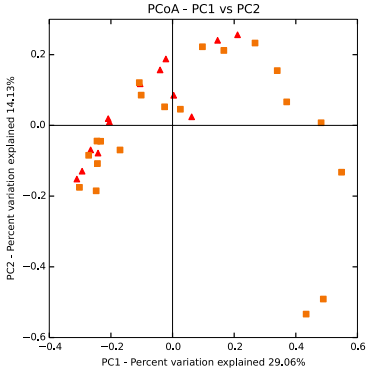
Supplemental table 7.1. Identification of species (feces)

	Peak_ID	Bacterial species	P value	FDR value	mean frequency healthy controls	mean frequency compensated
Feces	321_Firmicutes	*Streptococcus bovis/gallolyticus*	0,00	0,02	3,85	9,36
	322_Firmicutes		0,00	0,02	3,62	8,04
	298_Firmicutes	*Streptococcus mitis group*	0,00	0,02	1,54	5,68
	299_Firmicutes		0,00	0,02	1,12	4,00
	308_Bacteroidetes	Unclassified Bacteroidetes	0,00	0,02	10,85	5,28
	306_Bacteroidetes		0,00	0,02	9,15	4,20
Duodenum	264_Firmicutes	*Lactobacillus johnsonii*	0,00	0,15	0,50	3,23
	265_Firmicutes		0,00	0,16	0,30	3,46
	496_Firmicutes		0,00	0,15	0,25	2,23
	495_Firmicutes		0,00	0,15	0,15	2,31
	497_Firmicutes		0,01	0,15	0,35	1,77
	276_Firmicutes	*Enterococcus* spp.	0,00	0,14	1,85	4,77
	485_Firmicutes	*Lactobacillus plantarum*	0,00	0,15	0,45	2,23
	292_Firmicutes	*Staphylococcus spp.*	0,00	0,15	4,70	9,31
	289_Firmicutes		0,00	0,15	2,75	6,46
	366_Bacteroidetes	Unclassified Bacteroidetes	0,00	0,15	0,35	2,69
	368_Bacteroidetes		0,00	0,15	0,85	4,92
	369_Bacteroidetes		0,00	0,15	0,75	3,77
	367_Bacteroidetes		0,00	0,16	0,50	3,31

S7.2. Principal coordinate analysis (PCoA) on Bray Curtis dissimilarity of fecal samples between healthy controls (orange squares) and patients with compensated cirrhosis (red triangles) based on health status.



S7.3. Principal coordinate analysis (PCoA) on Bray Curtis dissimilarity of duodenal samples between healthy controls (orange squares) and patients with compensated cirrhosis (red triangles) based on health status.



SUPPLEMENTAL MATERIAL AND METHODS

DNA isolation

Frozen fecal samples were kept at 0°C, and 500 µl lysis buffer (Biomérieux, Marcy l'Etoile, France) was added to approximately 250 mg of the fecal samples. The samples were then vortexed and shaken for 5 min at room temperature (RT). Subsequently, the samples were centrifuged for 2 min at 14,000 rpm and 100 µl supernatant was used for the subsequent isolation procedure. Two ml lysis buffer (Biomérieux, Marcy l'Etoile, France) was added to the supernatant. The samples were incubated for 10 min at RT and 70 µl magnetic silica beads (Biomérieux, Marcy l'Etoile, France) were added. The DNA isolation was performed with the NucliSENS® easyMag® automated DNA isolation machine (Biomérieux, Marcy l'Etoile, France) with the Specific A protocol as described by the manufacturer. DNA was eluted in 110 µl elution buffer (Biomérieux, Marcy l'Etoile, France).

Duodenal and sigmoid biopsies were thawed on RT, and 360 µl ATL buffer (Qiagen, Hilden, Germany) and 40 µl proteinase K (Qiagen, Hilden, Germany) were added to each biopsy specimen. The samples were shaken at maximum speed for 1 hour at 56°C. Subsequently, 400 µl AL buffer (Qiagen, Hilden, Germany) was added and the samples were vortexed for 15 seconds and incubated for 10 min at 70°C. Samples were centrifuged for 2 min at 18,000 rcf and the complete supernatant was used for the subsequent isolation procedure. Two ml lysis buffer (Biomérieux, Marcy l'Etoile, France) was added to supernatants and the samples were incubated for 10 min at RT. Seventy µl magnetic silica beads (Biomérieux, Marcy l'Etoile, France) were added to each sample. The DNA isolation was performed with the NucliSENS® easyMag® automated DNA isolation machine (Biomérieux, Marcy l'Etoile, France) with the Specific A protocol as described by the manufacturer.

16S-23S IS profiling of the intestinal microbiota

IS-pro is a validated PCR based bacterial profiling technique,[17] which combines species differentiation by the 16S-23S rRNA intergenic spacer (IS) region with taxonomic classification by the phylum-specific fluorescent labeled primers. The IS-pro technique uses 2 multiplex PCRs to detect the phyla Firmicutes, Actinobacteria, Fusobacteria, Verrucomicrobia, Bacteroidetes and Proteobacteria. The first PCR reaction consists of 3 unlabeled reverse primers and 2 different fluorescently labeled forward primers (one specific for Firmicutes, Actinobacteria, Fusobacteria and Verrucomicrobia and one specific for Bacteroidetes). The second PCR consists of a combination of 7 reverse primers and one forward labeled primer specific for Proteobacteria. Amplification of the 16S-23S rRNA IS-regions was performed with the IS-pro assay (IS-Diagnostics, Amsterdam, the Netherlands) according to the protocol provided by the manufacturer on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA).[17] To analyze the DNA fragments, 5 µl PCR product was added to 19.8 µl formamide and 0.2 µl MapMarker 1000

ROX-labeled size marker (Bioventures, Murfreesboro, TN, USA). DNA fragment analysis was performed on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems).

8

General discussion

GENERAL DISCUSSION

The important role of the microbiota in health and disease, in combination with recent advances in next-generation sequencing (NGS) technologies, has instigated the number of studies investigating the microbiota composition. Though, this relatively young research field suffers from methodological limitations and lacks standardization, for example with regard to methods used for sample collection and microbiota analysis, especially in clinical studies. Standardization is needed since data from the microbiota composition are complex and can be affected by environmental conditions (*i.e.* temperature, oxygen level) and any bias introduced in the process from sample collection to data analysis, could significantly impact the observed microbiota composition. In this thesis, we focused on fecal sample collection and the application of various analytical and methodological approaches to profile the microbiota in gastrointestinal health and disease.

Influence of study procedures on the microbiota

Fecal and mucosal samples can be used to investigate the microbiota of the gastrointestinal tract. The latter are more difficult to obtain due to the need of invasive surgical or endoscopic procedures. Furthermore, the effect of procedure related factors, such as antibiotics (as commonly used prophylactic in selective decontamination of the digestive tract before surgical procedures) and bowel cleansing (as commonly used prior to a lower gastrointestinal (GI) endoscopy) on the microbiota, have to be considered (1–3). Although novel sampling strategies for example electronic capsules for delivery and sampling, such as the IntelliCap© system (4) are being developed to avoid these factors, invasive procedures are currently still necessary to assess the mucosa associated microbiota.

The fecal microbiota does not reflect the mucosal microbiota along the total GI tract, but is considered to reflect mainly the luminal microbiota of the distal intestinal tract and is therefore often used as a proxy for the intestinal microbiota (5,6). Fecal sampling is non-invasive and allows auto-collection at home, and is thereby suited for large-scale cohort studies and clinical settings. However, the microbiota composition may be affected by different sampling and storage procedures. In **Chapter 2**, we demonstrated that fecal samples stored at -20°C for 1 week, at +4°C or at room temperature for 24 hours maintained a similar bacterial structure as fecal samples immediately stored at -80 °C. The inter-individual variation exceeded the variation introduced by the different sampling and storage methods as all samples were found to cluster by test subject and not by the sampling or storage method. Based on these findings and the results of similar studies, we conclude that temperature (in refrigerator/freezer) or oxygen level up to 24 hours do not have a significant impact on the fecal microbiota composition (7–10). This will facilitate sample collection from consecutive patients in clinical practice. Nowadays, various collection devices with stabilization buffers are being developed (*e.g.* OMNIgene gut©

collection system), which could further facilitate microbiota studies in near future. However, high costs and the possibility to perform other '-omics' than metagenomics must also be taken into account (11). When setting up a study, we still recommend to use a single standardized sampling and storage method to limit any potential bias (7).

It must be noted that the above findings cannot be extrapolated to investigations of the microbiota activity (*e.g.* metabolites). Therefore, similar studies should be undertaken to investigate the impact of sample storage and processing on the meta-metabolome, metatranscriptome and metaproteome.

Apart from different sampling and storage procedures, the choice of the subsequent DNA extraction method, including disruption of the bacterial membranes as a first step, also has an impact on the observed microbial community structure. Considering the differences in bacterial cell wall structure and integrity, inadequate lysis can result in enrichment of DNA originating from bacteria that are more easily to disrupt (*e.g.* Gram-negatives). Although the cell wall structure of bacteria can be negatively affected by freezing samples prior to DNA extraction, mechanical disruption is necessary to obtain an unbiased representative DNA collection of the microbiota (12). Applying mechanical bead-beating during cell lysis results in an increase of DNA yield from bacteria that are difficult to disrupt (*e.g.* Gram-positives and spores). Furthermore, feces contain various components that can inhibit the PCR reaction (*e.g.* bile acids). For all studies performed in this thesis, these issues were addressed by combining mechanical (bead-beating) with chemical lysis and the use of commercial DNA isolation kits to remove PCR inhibitors. Still, it has to be noted that different protocols can have huge impact on study outcome and hinders comparisons between studies, as recently shown by Wesolowska-Andersen *et al* (13). The authors compared DNA extraction protocols of two major research consortia, the American Human Microbiome Project (HMP) and the European MetaHit project, which both used next-generation sequencing methods to assess the microbiota composition and their results were published in high impact journals (14,15). One of the most striking differences was the significantly higher Firmicutes/Bacteroidetes ratio when DNA extraction was done according to the MetaHit protocol as compared to the HMP protocol. To prevent this in future studies, further standardization of microbiota studies is warranted. Next to the technical issues, differences in sequencing approaches (16), sequencing technologies (17) and choice of bioinformatic tools (18) can affect the observed microbiota structure. Another challenge in microbiota data analysis is the problem of multiple comparisons, and therefore multiple testing correction is essential to reduce the chance of spurious findings (19).

The studies in the present thesis addressed the composition of the intestinal microbiota based on the 16S rRNA gene or 16S-23S interspace region, but did not evaluate the functional capacity of the microbiota. It is possible to infer the metagenomic function from the marker gene (16S

gene) by predictive metagenomics profiling (PMP) with PICRUSt or tax4fun (20,21). Some limitations exist when inferring the metagenomic function based on 16S sequencing, in particular the predictions depend on sequenced micro-organisms, and therefore in the absence of a reference micro-organism predictions on the metagenomics function cannot be made. Despite this limitation predictive metagenomics profiling is rather cost-effective to decide whether to proceed with investigating the functional changes of the microbial community and eventually which experimental strategy should be used. As the genetic capacity not necessarily reflects the actual activity of the microbiota, additional 'omics' approaches, such as metatranscriptomics, metaproteomics and meta-metabolomics to investigate the biological significance of the microbiota, can be undertaken (22).

In addition to bacteria and Archaea, the microbiota consists of microbial eukaryotes (*e.g.* funghi) and viruses (including bacteriophages) that are not detected by the sequencing methods employed in this thesis. These organisms can be detected by other methods such as targeting a different gene or genomic region for the detection of funghi (18S rRNA gene and the internal transcribed spacer (ITS)). For the characterization of microbial eukaryotes, the bioinformatics pipeline is similar to the pipelines for bacterial marker genes, but protocols are not as standardized as for 16S rRNA gene analysis. Investigating the human viriome is even more challenging since no gene or genomic region is homologous across all viruses. To bypass this problem, virus like particles (VLP) can be isolated by size fractionation and subsequently sequenced using metagenomics or DNA micro-arrays (23). The viriome and eukaryotic component of the microbiota remains relatively unexplored, but is important in future studies to obtain a complete picture of the microbiota and its implications for health and disease.

Influence of patient characteristics on the microbiota

Previous studies revealed that the microbiota is unique for each individual and its composition does not only depend on genetics, early lifestyle factors or demographics (*e.g.* gender, age, BMI, pregnancy), but also on recent events such as smoking, dietary patterns or medication use (24–28). For example, antibiotics have a prominent effect on the microbiota, which is most pronounced when antibiotics were taken recently. Therefore, most studies only include subjects that have not used antibiotics at least a certain period prior to fecal collection, although the chosen time windows are rather arbitrarily and vary between studies from a few days to a few months prior to sample collection (22). Depending on the antibiotic class and dosage used, it may take six months to four years for the microbiota to recover (29). Also proton pump inhibitors (PPIs), metformin and thiopurines have been reported to be associated with changes in the microbiota (30–32). As the use of these drugs could not be used as exclusion criteria, we checked their impact in our studies (**Chapters 3, 4, 6 and 7**). We did however not find these medications to affect the microbiota composition, although it should be noted that the groups of users were rather small.

The effect of baseline demographics such as ethnicity on the microbiota is hard to unravel, since other factors such as diet, hygiene and life-style (rural versus urban) are often coherent to ethnicity. It seems that ethnicity plays only a limited role in shaping and influencing the microbiota. For example, diet was found to have a larger effect on the microbiota then ethnicity (33). Major changes in dietary patterns can affect the microbiota as demonstrated by a recent dietary intervention study including African American and rural African participants (34). Furthermore, also BMI, age and prior surgery has to be taken into account. Stool consistency can also vary considerably between subjects, especially in those with an intestinal disorder. This factor is often not reported, but may impact the microbiota composition and activity (35). A recent study reported that stool consistency correlated negatively with species richness, and positively with the Bacteroidetes/Firmicutes ratio and *Akkermansia* and *Methanobrevibacter* abundances (36). We did not find an effect of stool consistency on the effect of sampling and storage of fecal samples, as reported in **Chapter 2**. However, to prevent any potential confounding, we checked for the effect of fecal consistency using the Bristol Stool Scale Chart or by the fecal protein concentration.

Study designs

Many publications on the microbiota in health and disease are based on cross-sectional studies and often do not take potential confounders into account. Although the results of such studies yielded important information, they are also hampered by some major limitations. Cross-sectional studies fail for example to determine whether microbial shifts are a cause or consequence of the disorder, which is further hindered by alterations in disease course over time. For example, IBD patients have a relapsing disease course and often undergo surgery and/or develop intestinal complications (*e.g.* fistula, stenosis), which could alter the microbiota composition.
The temporal variability is also an aspect that should be considered in microbiota studies and has been linked to microbiota diversity. For example, a less diverse microbiota was found to be more prone to temporal variability as compared to a highly diverse microbiota (37). The microbiota is also known to have a large inter-individual variation. These two aspects can be taken into account when using longitudinal study designs. Studies in which patients are their own control are therefore valuable to discover disease-specific microbial shifts for example in the symptomatic and asymptomatic stage of a disease.

Regardless of the different methodologies or approaches used to study the microbiota, a couple of fundamental aspects should be kept in mind: 1) confounding factors need to be minimized when designing the study or controlled for in the statistical analysis, 2) consistent experimental and analytical methods should be applied throughout the entire study and 3) all relevant metadata should preferably be documented prospectively. Furthermore, it is important to keep detailed records of the bioinformatic steps of all performed analyses and to deposit all data in public databases, allowing further comparisons and analyses.

The intestinal microbiota in disease etiology and as diagnostic tool

Up to now, mainly cross-sectional studies have described intestinal microbiota perturbations in patients with manifested diseases, including Crohn's disease, liver cirrhosis and obesity as compared to healthy controls. Such studies can however not disentangle whether these perturbations are already present prior to disease onset, being a potential cause of the disease, or merely a consequence of the disease or associated treatments. Prospective cohort studies are therefore warranted to link these microbiota alterations with disease and symptom onset. Ideally, these prospective studies need to integrate microbiome, metagenome, metatranscriptome and meta-metabolomics data with clinical and environmental data to move from associations to causation (38). Despite all the effort that has been put into designing the perfect or most optimal human microbiota study, it still remains difficult to establish a true causality. This is in part due to the individuality of the human microbiota, but also due to variables and factors that are often not possible and/or ethical to control for (*e.g.* exact disease onset and/or medication use). Furthermore, the host-microbe interactions and functionality of the key microbial players identified in observational studies should also be further investigated. *In vitro* and *in vivo* models are therefore an essential supplement to human based/clinical research in the investigation to the role of the microbiota in health and disease.

The best empirical evidence that the microbiota can drive disease comes from experiments in which the microbiota from diseased donors and controls is transplanted into healthy germ-free hosts. When recipients of the disease-associated microbiome display the disease, the microbiota is considered to play a causal role (39). For example, previous studies have shown that liver cirrhosis, colitis and obesity can all be transmitted by microbiota transplantations (39–41). We confirmed an altered microbiota composition in patients with liver cirrhosis (**Chapter 7**), obesity (**Chapter 6**) and Crohn's disease (**Chapter 3**) when compared to healthy controls. To further unravel the pathophysiological relevance of the observed perturbations, we evaluated the impact of several host factors on the microbiota structure. In overweight patients for example (**Chapter 6**), we found that the baseline microbiota composition correlated significantly with hsCRP, which is in line with a (low grade) systemic inflammation in these subjects (42). However, after weight reduction by sleeve gastrectomy, no correlations were found with hsCRP. The (long-term) relevance of the post-surgery alterations, which did not move towards a more lean profile, should therefore be further investigated in future studies.

In Crohn's disease (**Chapter 5**), we linked the metabolome profile (*i.e.* volatile organic compounds) in exhaled air to the microbiota composition. VOCs can be affected by both host processes such as inflammation, oxidative stress and hepatic function, as well as by the intestinal microbiota. Interestingly, we found a strong correlation between exhaled VOCs and the microbiota composition. On one hand, VOCs in exhaled air may provide an interesting non-invasive tool for monitoring the microbiota activity, but integrating VOCs and microbiota may

also provide interesting leads for underlying pathophysiological mechanisms and possible interventions. Altered VOC profiles have also been shown for example in patients with liver cirrhosis and IBS, and it may be interesting to further investigate possible correlations in these patients as well. A better understanding of the relation between the intestinal microbiota and disease might enable us to develop personalized therapies. The identification of specific host/environment-microbe interactions could lead to novel therapeutic recommendations and life style advice based on the microbiota profile or bacteria-related metabolites. Pharmacomicrobiomics may also become a part of personalized medicine since the efficacy and toxicity of drugs can be affected by the intestinal microbiota. For example, the intestinal microbiota is able to convert the pro-drug thioguanine (IBD medication) into the active metabolite 6-thio-GTP (43). Thereby, the microbiota may contribute to the effect of thiopurines in the treatment of IBD patients. Another example might be the application of personalized antibiotic therapy by screening the microbiota for antibiotic resistance genes prior the start of antibiotic therapy in order to decrease the likelihood of treatment failure and to reduce the occurrence and dissemination of antibiotic resistance.

Although cause and consequence are often not clear, several studies showed differences in the microbiota composition of diseased versus healthy subjects. Therefore, we checked whether microbial profiling can be used as a diagnostic tool. We found that the fecal microbiota may have potential as disease progression marker for patients with liver cirrhosis (**Chapter 7**) and to assess disease activity in patients with Crohn's disease (**Chapter 3 and 4**). Although both findings should be validated in further studies, they are of interest as the observed changes may be linked to disease etiology. Therefore, it would be interesting to further investigate the potential of microbiota profiling for the early identification of patients at risk for disease progression. Novel diagnostic or monitoring tests based on the microbiota should be reproducible, high-throughput, affordable and include a relatively simple sample collection. Furthermore, test results should be readily interpretable and relevant to the disease. Despite all efforts that have been put into research, there are still challenges with the abovementioned factors. The emergence of next-generation sequencing technologies results in a large amount of information, but the establishment of correlations between microbiota patterns and specific diseases remains challenging. New diagnostic standards should be adopted since microbiota data analysis represents patterns of different taxa rather than a single bacterium. This has also been demonstrated in the current thesis (**Chapter 4**), where we show that a combination of microbial taxa rather than one single taxon, could be used as a disease marker in Crohn's disease patients. Furthermore, analysis of microbiota data is time-consuming and requires expert bioinformaticians. The techniques that have been used to investigate the microbiota (*e.g.* Illumina sequencing) are rather complex for routine analysis of single patient samples. **In Chapter 7** we used IS-pro, a validated microbiota profiling method that is relatively easy to implement in clinical practice, enables analysis of multiple or individual samples and the interpretation of the

results is straight forward. As the importance of the role of the intestinal microbiota in health and disease becomes more established and recognized, it is expected that microbiota profiling will become an important diagnostic and prognostic tool, allowing health care professionals to assess their patients' condition more easily and accurately in the future.

Future perspectives

There are still many challenges that have to be overcome before microbiota analyses will become part of routine diagnostics in daily clinical practice. For most diseases, associations with the intestinal microbiota have been shown, but detailed knowledge on the pathophysiological relevance is largely lacking. Moreover, human intervention studies targeting the intestinal microbiota and thereby host outcome, for example by probiotics, are still of limited value for example due to inconsistent results, limited efficacy and/or generic approaches. More insight is needed in key microbes and/or metabolic pathways involved in certain diseases to enable more tailored approaches.

To take microbiota studies to a next level, standardized and state of the art technologies in combination with advanced integrated analyses of multidimensional datasets are needed. By conducting high-quality scientific studies on the microbiota and validating previous findings, we can achieve a better understanding of disease, develop more accurate and patient-friendly diagnostic methods and devise personalized treatment options. Future microbiota studies should not only explore the alpha and beta diversity, but also potential interactions between microbes by means of network analysis since microbes comprising the microbiota exist as a complex and interactive community rather than independent microbes. This should include the composition and activity of the microbiota, as well as host factors. Furthermore, future microbiota studies should be driven by specific questions relevant to the clinical aspects and the nature/progression of the disease and should make use of experimental models to further unravel underlying mechanisms. Therefore to fully understand the relationship between the microbiota and the human physiology, collaborations between clinical and basic science is necessary to understand the microbiota information in the context of clinical metadata.

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9

Summary

SUMMARY

This thesis aimed to investigate the role of the intestinal microbiota in Crohn's disease, liver cirrhosis and (morbid) obesity and explored its potential as a disease progression marker by applying various molecular, epidemiological and statistical approaches.

Large-scale cohort studies are essential to investigate the role of the intestinal microbiota in the onset and progression of diseases. Sample collection in patient cohorts is however challenging as on one hand (repeated) samples need to be collected in such a way that microbial integrity is being maintained between defecation and sample processing, while on the other hand this sample collection needs to be logistically feasible and implemented in the daily clinical routine. To address these challenges, the effect of different sampling and storage methods on the fecal microbiota composition of healthy individuals and patients with gastrointestinal disorders associated with microbial perturbations and altered bowel habits was investigated in **Chapter 2**. Fecal samples of healthy individuals, IBS and IBD patients were aliquoted immediately after defecation and aliquots were stored directly at -80°C, at -20°C for 1 week, at +4°C for 24 hours or at room temperature for 24 hours before subsequent storage at -80°C. A commercially available transport swab with a preservation medium was also collected from the fecal sample and stored at room temperature for 48-72 hours before freezing. Comparisons between feces immediately stored at -80°C and feces handled according to the other sampling and storage methods showed no differences in bacterial richness and diversity, regardless of differences in fecal consistency. Only the fecal swab showed a significant higher bacterial richness and diversity and a higher abundance of *Ruminococcus* and Enterobacteriaceae. Despite these differences, when comparing the overall microbial community structure between samples, samples still clustered together based on the test subject and not based on the sampling or storage method, indicating that the effect of the employed sampling and storage method is limited. Based on these results, we demonstrate that fecal samples stored at -20°C for 1 week, at +4°C for 24 hours and at room temperature for 24 hours maintained a similar bacterial structure as fecal samples immediately stored at -80 °C. Still we recommend using a single sampling and storage method within a study to prevent potential bias.

Subsequently, the microbiota composition was investigated in three diseases, starting with Crohn's disease. Intestinal microbial alternations have been reported not only between Crohn's disease patients and healthy individuals, but also between Crohn's disease patients during remission and exacerbations. This suggests that the microbiota may play a role in both the development and progression of Crohn's disease and supports its potential use as a disease activity marker. In **Chapter 3** we investigated the role of the intestinal microbiota in relation to disease activity in Crohn's disease in a prospective follow-up study. The stability and microbiota structure was compared between inactive patients maintaining remission versus those develop-

ing an exacerbation over time. We observed a stronger temporal microbiota stability in healthy individuals as compared to CD patients regardless whether these patients had a stable disease course or developed an exacerbation over time. Notably, a subgroup of the CD patients had a similar microbial composition as healthy individuals characterized by a high microbial diversity and a high relative abundance of *Faecalibacterium prausnitzii*.

In **chapter 4** we conducted a proof-of-principle study to investigate whether the fecal microbiota could be used as a disease activity marker by applying machine learning techniques. We used random forest to find a combination of bacterial taxa that could discriminate between Crohn's disease patients during remission and exacerbation and identified a set of 50 Operational Taxonomic Units (OTUs) that was able to correctly predict 73% of remission and 79% of active samples with an AUC of 0.82. These results support the potential of the fecal microbiota as a non-invasive method to monitor disease activity in Crohn's disease patients.

While **Chapters 3** and **4** focused on the presence of the microbiota, recent studies revealed the importance of microbial metabolism for the host and demonstrated how these metabolites, such as volatile organic compounds (VOCs), are indicative for different (sub-clinical) diseases. The microbiota produces VOCs that cannot only be measured in feces, but also in exhaled breath. In **Chapter 5** we explored the relation between the intestinal microbiota composition and volatile metabolites in breath of Crohn's disease patients in remission and during an exacerbation, and constructed the metabolic pathways that are associated with the relations found between the VOCs and microbiota. We observed a strong correlation between 19 bacterial taxa and 18 VOCs from CD patients during active disease and also observed a strong correlation between 17 bacterial taxa and 17 VOCs in CD patients in remission. Of these VOCs and bacterial taxa, nine VOCs and three bacterial taxa were found to be overlapping between active CD and CD in remission. Furthermore, we found specific VOCs to be correlated with the relative abundance of specific bacterial taxa. This study is the first study demonstrating a strong relation between the microbiota and VOCs in exhaled breath. Elucidating this correlation is warranted to increase our understanding on the functional effects of the microbiota, which might eventually lead to microbiome targeted interventions or disease monitoring.

In **Chapter 6** we investigated the impact of sleeve gastrectomy (SG) on the fecal microbiota of obese individuals, whether this bariatric surgical procedure changed the obese microbiota towards a lean profile, and examined whether the microbial alterations upon SG could be linked to markers of inflammation, intestinal barrier function and glycemic control. Fecal samples were collected from obese individuals prior to and at two and six months after the bariatric surgery. Furthermore, feces was also collected from a lean control group. The microbiota composition of obese individuals differed significantly from that of lean individuals. A correlation between the microbiota and plasma hsCRP levels of obese patients prior to SG was also found, but no

correlations were found between the microbiota composition and markers for glycemic control, intestinal inflammation, intestinal permeability and fecal consistency. Although we observed some time specific increases of bacteria (*e.g. Staphylococcus spp* after two months), overall alterations of the microbiota composition were consistent two and six months after SG, indicating that these microbial shifts are not a short-term effect of the procedure or antibiotic prophylaxis, but presumably the result of SG induced changes in the gastrointestinal physiology. The microbiota did however not change towards a lean profile post-SG, but developed towards a distinct microbiota, mainly driven by facultative anaerobic bacteria, such as *Streptococcus, Actinomyces* and *Rothia*, that are characteristic for the upper gastrointestinal tract. However, whether and to what extend these microbial shifts have an effect on the host physiology and metabolic improvements is not clear and should be investigated further.

Studies in the previous chapters assessed the microbiota by means of next-generation sequencing which is not readily applicable in the clinic yet. In **Chapter 7,** we used Interspacer-profiling (IS-pro), a novel, rapid and easily implementable technique for microbiota profiling, to investigate the effect of liver cirrhosis on the microbiota and to evaluate the potential of the microbiota as a disease progression marker as indicated by the Child-Pugh class. We also investigated whether intestinal permeability markers were linked with the microbiota since changes in the intestinal microbiota and an increased permeability could contribute to bacterial translocation and thereby to disease progression. This study included feces as well as sigmoid and duodenum biopsies of healthy individuals and compensated liver cirrhosis patients, and feces of decompensated cirrhotic patients. We observed a significantly higher microbial richness and diversity within the phylum of Firmicutes and a lower richness for Bacteroidetes in feces of compensated and decompensated liver cirrhosis patients as compared to healthy individuals. The observed differences in microbial richness and diversity between the study groups were more pronounced as disease progresses, suggesting the possibility to use the fecal microbiota as a marker for disease progression. For the duodenum biopsies, only an increased richness for Firmicutes was found in compensated cirrhosis patients as compared to healthy individuals. Remarkably no significant microbial differences were found in the sigmoid biopsies. A positive correlation between *Lactobacillus johnsonii* and L/R ratio (small intestinal permeability) was also found. Constrained ordination analysis including fecal samples of all study subjects showed distinct fecal clusters based on the disease severity as scored by the Child-Pugh classification, but not on demographics, etiology or medication. This supports the concept that microbial profiling of the fecal microbiota can be used to monitor disease progression in liver cirrhosis.

9

Samenvatting

SAMENVATTING

In dit proefschrift is de rol van de darmmicrobiota bij de ziekte van Crohn, levercirrose en (morbide) obesitas onderzocht en is gekeken of de darmmicrobiota kan worden gebruikt als een marker voor ziekte en ziektebeloop. Hierbij is gebruik gemaakt van verschillende moleculaire, epidemiologische en statistische methoden.

Grote cohortstudies zijn onmisbaar om de rol van de darmmicrobiota te onderzoeken bij het ontstaan van de eerste symptomen alsook het ziektebeloop. Het verzamelen van ontlastingsmonsters bij patiënten vormt een grote uitdaging. Deze moeten zodanig verzameld worden dat de microbiële samenstelling behouden blijft vanaf het moment van defecatie tot het moment van verwerking van de monsters. Tegelijkertijd moet de manier waarop ontlastingsmonsters worden verzameld toepasbaar zijn in een klinische setting. Daarom is in **Hoofdstuk 2** onderzocht wat het effect is van verschillende verzamelings- en opslagmethodes op de microbiota samenstelling in ontlastingsmonsters van gezonde individuen en patiënten die lijden aan darmziekten die geassocieerd zijn met veranderingen in de microbiota en stoelgang (patiënten met het Prikkelbare Darm Syndroom (PDS) en chronische Inflammatoire darmziekten (IBD)). Direct na defecatie werd het fecaal materiaal verdeeld en onder vier verschillende condities opgeslagen (direct bij -80°C , een week bij -20°C , 24 uur bij +4°C en 24 uur bij kamertemperatuur). Daarna werden de monsters allen opgeslagen bij -80°C tot het moment dat de monsters werden verwerkt. Daarnaast werd ook een commerciële wattenstok onderzocht, met een bewaarmedium ontwikkeld om opportunistische darmpathogenen te behouden. Deze wattenstokken werden op kamertemperatuur bewaard gedurende 48-72 uur voordat ze werden opgeslagen bij -80°C . Ondanks de verschillen in fecale consistenties, werd er geen verschil gevonden in het aantal bacteriesoorten (bacteriële soortenrijkheid) en de bacteriële diversiteit tussen de ontlastingsmonsters die direct werden opgeslagen bij -80°C en feces die een week bij -20°C , 24 uur bij +4°C of 24 uur bij kamertemperatuur waren bewaard. Ontlasting die werd verzameld met behulp van de commerciële wattenstok toonde echter een significant hogere bacteriële soortenrijkheid en diversiteit en een grotere hoeveelheid van *Ruminococcus* en Enterobacteriaceae vergeleken met ontlasting die direct bij -80°C werd opgeslagen. Ondanks dit verschil is het effect van de verschillende verzamelings- en opslagmethodes op de microbiota samenstelling beperkt, aangezien alle monsters groeperen op basis van de individuele proefpersoon en niet op basis van de verschillende verzamelings- en opslagmethodes. In dit onderzoek hebben we laten zien dat de microbiota samenstelling in ontlastingsmonsters die een week bij -20°C, 24 uur bij +4°C of 24 uur bij kamertemperatuur zijn bewaard, niet noemenswaardig verandert ten opzichte van monsters die direct bij -80°C werden opgeslagen. Desalniettemin adviseren we voor elke studie één verzamelings- en opslagmethode te kiezen om eventuele invloed te voorkomen.

In de volgende studies werd de microbiota onderzocht in drie verschillende ziektes, de ziekte van Crohn, obesitas en levercirrose. Diverse studies hebben aangetoond dat de darmmicrobiota van patiënten met de ziekte van Crohn anders is dan die van gezonde individuen, maar ook verschilt tussen patiënten in remissie en tijdens een exacerbatie. Dit suggereert dat de microbiota mogelijk een rol kan spelen in het ontstaan alsook het verdere beloop van de ziekte van Crohn en wijst op de mogelijkheid om de microbiota te gebruiken als een marker voor ziekteactiviteit. In **Hoofdstuk 3** onderzochten we de rol van de darmmicrobiota in relatie tot ziekteactiviteit in een prospectief *follow-up* cohort waarbij de stabiliteit en structuur van de microbiota vergeleken werd van patiënten in remissie ten opzichte van patiënten die tijdens de follow-up een exacerbatie ontwikkelden. De fecale microbiota van gezonde individuen was stabieler over de tijd in vergelijking met die van Crohn patiënten, ongeacht of deze patiënten in remissie bleven of een exacerbatie ontwikkelden gedurende de studieperiode. Er was geen verschil in de microbiota stabiliteit tussen Crohn patiënten die in remissie bleven en Crohn patienten die een exacerbatie ontwikkelden. Wel was het opvallend dat de microbiota samenstelling in een subgroep van de Crohn patiënten vergelijkbaar was met de microbiota samenstelling van gezonde individuen. De microbiota van deze subgroep Crohn patiënten had een hogere microbiota diversiteit en een grotere relatieve hoeveelheid *Faecalibacterium prausnitzii* vergeleken met de overige Crohn patiënten.

Om te onderzoeken of de fecale microbiota kan worden gebruikt als een marker voor ziekteactiviteit in Crohn patiënten, hebben we in **Hoofdstuk 4** onderzocht of op basis van een combinatie van fecale bacteriële taxa een onderscheid kon worden gemaakt tussen patiënten in remissie en patiënten tijdens een exacerbatie. Door het gebruik van *machine learning* technieken kon een set van 50 Operational Taxonomic Units (OTUs) geïdentificeerd worden die de twee groepen Crohn patiënten kon onderscheiden. Op basis van deze set OTUs was het mogelijk om 73% van alle remissie ontlastingsmonsters en 79% van alle exacerbatie ontlastingsmonsters correct te voorspellen met een AUC van 0.82. Dit resultaat geeft aan dat de fecale microbiota de potentie heeft als een niet invasieve methode om ziekteactiviteit bij patiënten met de ziekte van Crohn te monitoren.

De **Hoofdstukken 3** en **4** waren gericht op de microbiota samenstelling, maar recent onderzoek heeft ook laten zien dat metabolieten van micro-organismen, zoals bijvoorbeeld vluchtige organische verbindingen (VOCs), van invloed zijn op de gastheer. Diverse studies hebben aangetoond dat VOC-profielen kunnen worden gebruikt om diverse darmziektes aan te tonen, maar ook om ziekteactiviteit in IBD te monitoren. De VOCs die de microbiota produceert kunnen niet alleen maar worden gedetecteerd in ontlasting, maar ook in uitgeademde lucht. In **Hoofdstuk 5** onderzochten we de relatie tussen de fecale microbiota samenstelling en VOCs in uitgeademde lucht van Crohn patiënten in remissie en tijdens een exacerbatie. Uiteindelijk vonden we een sterke correlatie tussen 19 bacteriële taxa en 18 VOCs in Crohn patiënten gedurende een

exacerbatie en tussen 17 bacteriële taxa en 17 VOCs in Crohn patiënten in remissie. Verder vonden we specifieke VOCs die gecorreleerd waren met specifieke bacteriële taxa. Deze studie is de eerste in zijn soort waarbij een sterke correlatie is aangetoond tussen de microbiota in de darm en VOCs in uitgeademde lucht. Het verder onderzoeken van deze correlaties is zeer waardevol om meer informatie te verkrijgen over de functionele effecten van de microbiota. Deze kennis zou kunnen leiden tot nieuwe methodes om ziektes te monitoren en interventies die aangrijpen op het microbioom.

In **Hoofdstuk 6** onderzochten we het effect van bariatrische chirurgie, te weten *sleeve gastrectomie* (SG) op de fecale microbiota van mensen met obesitas. Hierbij was het doel te kijken of deze gewichts-reducerende ingreep de microbiota op dusdanige manier verandert dat deze meer lijkt op de microbiota van niet-obesen. Hierbij werd ook onderzocht of de microbiota veranderingen 2 en 6 maanden na de ingreep, correleerden met veranderingen in markers voor inflammatie (plasma hsCRP en fecaal calprotectine), intestinale barrière functie (suikerratio's in urine) en glycemische index (HbA1c-waarde in plasma hetgeen de gemiddelde bloedsuikerspiegel van voorafgaande weken reflecteert). Ontlastingsmonsters werden verzameld van mensen met obesitas voor de SG en twee en zes maanden na SG. Ook werd er eenmalig ontlasting verzameld van een controle groep bestaande uit gezonde mensen met een normaal gewicht. De samenstelling van de microbiota was anders in de obsese personen (pre-chirurgie)) ten opzichte van gezonde personen. Ook werd er een correlatie gevonden tussen de microbiota samenstelling en plasma hsCRP in mensen met obesitas pre-chirurgie, maar deze correlatie werd niet gevonden in gezonde personen en mensen met obesitas na SG. Er werd geen correlatie gevonden tussen de microbiota samenstelling en markers voor de glycemische index, darmontsteking, darmpermeabiliteit en consistentie van de ontlasting. Na SG, veranderde de microbiota samenstelling. De meeste veranderingen in microbiota samenstelling waren consistent twee en zes maanden na SG. Dit suggereert dat de veranderingen in de microbiota samenstelling geen tijdelijk gevolg zijn van de SG-procedure of het gebruik van antibiotica profylaxe, maar dat deze waarschijnlijk het gevolg zijn van de veranderingen in de gastro-intestinale fysiologie, veroorzaakt door de SG procedure. De veranderingen in de microbiota samenstelling van obese individuen na SG resulteerde niet tot een microbiota samenstelling vergelijkbaar met de microbiota van de controle groep. De microbiota samenstelling na SG werd gekarakteriseerd door een toename in facultatief anaerobe bacteriën zoals *Streptococcus, Actinomyces* en *Rothia,* die normaal te vinden zijn in de mondholte en het bovenste maag-darm stelsel. Het is nog onduidelijk of en in hoeverre deze veranderingen een effect hebben op de fysiologie van de gastheer, hetgeen moet worden onderzocht in vervolgonderzoeken.

De studies zoals beschreven in de **hoofdstukken 1** tot en met **6** brachten de microbiota samenstelling in beeld door gebruikt te maken van *next-generation-sequencing* technieken die nog niet toepasbaar zijn in de kliniek. In **hoofdstuk 7** gebruikten we de *Interspacer-profiling*

(IS-pro) techniek, een nieuwe en snelle techniek om de microbiota te profileren. Deze techniek is bovendien gemakkelijker te implementeren in de dagelijkse klinische praktijk. IS-pro werd gebruikt om het effect van levercirrose op de microbiota te bestuderen en om de mogelijkheid te onderzoeken of de microbiota kan worden gebruikt als marker voor de progressie van levercirrose, zoals geclassificeerd door middel van de Child-Pugh scores. Daarnaast hebben we onderzocht of er een relatie aanwezig was tussen markers voor darmpermeabiliteit en de microbiota, omdat veranderingen in de microbiota en een verhoogde permeabiliteit kunnen bijdragen aan de translocatie van bacteriën door de darmwand en daarmee kunnen bijdragen aan de verergering van het ziektebeloop. Voor deze studie hebben we zowel ontlasting alsook, sigmoïd en duodenum biopten afkomstig van patiënten met gecompenseerde levercirrose en gezonde individuen onderzocht. Daarnaast hebben we ook ontlastingsmonsters verzameld van gedecompenseerde levercirrose patiënten. De bacteriële soortenrijkheid en diversiteit van de Firmicutes was significant hoger in gedecompenseerde en gecompenseerde levercirrose patiënten in vergelijking met gezonde individuen, terwijl de bacteriële soortenrijkheid van de Bacteroidetes significant lager was in de lever cirrose patiënten in vergelijking met gezonde individuen. De verschillen in bacteriële soortenrijkheid en diversiteit waren meer uitgesproken naarmate de levercirrose verergerde. Opvallend was dat er geen verschil werd gevonden in de microbiota samenstelling van de sigmoïd biopten tussen de verschillende studiegroepen. Wel werden duidelijke verschillen gevonden in de duodenum biopten die in lijn waren met de bevindingen van de fecale microbiota. Ook werd een positieve correlatie gevonden tussen *Lactobacillus johnsonii* en de Lactulose/Rhamnose ratio (marker voor de dunne darm permeabiliteit). Uit de redundantieanalyse die uitgevoerd werd op de ontlastingsmonsters van alle proefpersonen, bleken alle monsters te clusteren op basis van de Child-Pugh classificatie en niet op demografie, etiologie of medicatie gebruik. De resultaten uit deze studie ondersteunen dat het profileren van de fecale microbiota kan worden gebruikt om het verloop van levercirrose te monitoren.

9

Valorisation

VALORISATION

Relevance for society and economy

Chronic disorders, such as obesity, liver cirrhosis and inflammatory bowel disease (IBD; primarily including Crohn's disease and ulcerative colitis), account for nearly two-thirds of deaths world-wide and pose a considerable financial burden to health care and society [1]. For obesity, costs are estimated at $190 billion in 2005 in the USA and expected to increase by $48-66 billion/year in 2030 [2]. For liver cirrhosis and chronic liver diseases costs are estimated at $2.5 billion USD/year in USA [3], while for ulcerative colitis the direct costs are estimated at $4 billion/year in the USA [4] and for Crohn's disease costs are estimated at $30 billion annually in the USA and Europe [5]. Furthermore, these disorders are expected to increase in the years to come.

Obesity rates are currently rising worldwide, especially in low- and middle income countries [6]. The number of children who are overweight or obese has nearly doubled from 5.4 million in 1990 to 10.6 million in 2014. There are more obese than underweight individuals in almost every region of the world (except for parts of sub-Saharan Africa and Asia) and comorbidities (*e.g.* cardiovascular diseases and diabetes) are responsible for more than 2.5 million deaths per year world-wide [7]. A major goal of obesity treatment is weight loss to limit or prevent complication, which can be achieved by lifestyle changes (although relatively ineffective with regard to long term weight loss) and bariatric surgery [6].

The prevalence of liver cirrhosis is also expected to rise worldwide. In 2010, it was estimated that liver cirrhosis is the cause of one million deaths worldwide (about 2% of all deaths) [8]. However, estimations of the exact prevalence are difficult, especially as compensated cirrhosis, the first stage of liver cirrhosis without complications, may not always be diagnosed [9,10]. Fatty liver disease, viral infections (hepatitis) and alcohol abuse are well-known causes of liver cirrhosis. The obesity epidemic discussed earlier is associated with the metabolic syndrome and non-alcoholic fatty liver disease (NAFLD), which are conditions that also contribute to the expected rise of patients progressing into liver cirrhosis in the future.

Crohn's disease and ulcerative colitis, also known as IBD, are generally considered to be 'Western' diseases, but the incidence is emerging worldwide in line with westernization [11]. This contributes to a high prevalence of IBD, which is also affected by the chronicity of the disease, young age of onset and low mortality. The health care system needs to be prepared for the rise of IBD patients in general, but also for the increase of the ageing IBD population with its more complex comorbidities [11,12]. Over the last decade, the treatment goal shifted from improvement of clinical symptoms towards achieving mucosal healing and prevention of long term complications, facilitated by the introduction of biological agents (*i.e.* anti-TNF) [13]. Since the demand for these expensive drugs is emerging, the high costs of these new treatments,

predicting treatment response and selecting the right treatment for the right person are an ongoing topics of debate.

Next to direct health care costs, the above mentioned diseases also have significant impact on the quality of life of the patients and indirect costs. Considering the current state, further research to decrease disease burden and improve current treatment options are warranted. On one hand this involves further insight in the pathophysiology and on the other hand non-invasive markers for early diagnosis and monitoring of disease course are urgently needed. All of the three mentioned disorders can be characterized by a complex multifactorial pathophysiology, including *e.g.* changes in the intestinal microbiota composition. Further studies on these microbial changes are needed to explore whether the microbiota can be used as a diagnostic or disease progression marker and to gain further insight in the mechanisms involved in onset and progression of these disorders.

Target groups

In this thesis, we focused on the microbiota of three different disorders, namely obesity, liver cirrhosis and Crohn's disease. Not only basic scientist and clinicians, but also companies that are developing novel diagnostic methods or intervention therapies based on the microbiota profiles may benefit from the results presented in this thesis. Furthermore, the results of the sampling and storage study in the second chapter are of relevance for scientists and clinicians to determine which sampling and storage methods should be used when setting up microbiota studies. The high direct (medical) and indirect (loss of work productivity) costs associated with the respective disorders also affects the general population. The results of this thesis could benefit the general population since the results might accelerate the development of novel diagnostic methods and treatments that are able to mitigate the financial burden of these disorders.

Innovations and activities

Since the microbiota plays an important role in health and disease, it should not come as a surprise that microbial changes have been associated with a plethora of various diseases and even different disease stages. This discovery has drawn attention of various scientists and companies to utilize the microbiota for diagnostic purposes or to develop microbiota targeted treatments and resulted in increasing effort in the microbiota research.

In this thesis, we have demonstrated that the microbiota composition in patients with CD, obesity or liver cirrhosis is different from controls. In general, a lower microbial diversity was found between patients with CD, liver cirrhosis and individuals with obesity as compared to controls. Although a loss of microbial diversity is often found in diseased individuals, a general conclusion or statement about the role of microbial diversity in health and disease can not be made since different types of host-microbe interactions are involved in the pathophysiology

of different diseases [14]. A higher abundance of SCFA producing genera (*Eubacterium, Dorea, Ruminococcus* and *Blautia*) was found in obese as compared to lean individuals. This observation supports the concept of an "obese" microbiota that might stimulate obesity development via increasing the energy harvest from diet.

Furthermore, we showed that not a single bacterial group or species, but a set of 50 bacterial taxa was found which can discriminate between active CD and CD in remission. Disease severity was found to have a pronounced impact on the microbiota composition in patients with liver cirrhosis. These data show the potential to use fecal microbiota data for disease monitoring. These findings need to be confirmed in further (prospective) studies, including the longitudinal, follow-up of patients.

Since the current state of the art technology based on next generation sequencing is not easy to implement in daily clinical practice, we applied a microbiota profiling technique (IS-pro) in the liver cirrhosis study. We demonstrated that IS-pro is a promising tool for noninvasive monitoring in clinical care and can be used for various microbiota-associated diseases.

In addition to microbiota-based disease monitoring or diagnostics, the microbiota may also have potential to be used to predict treatment responses. This is especially valuable for treatments that are known to be effective only in smaller percentages or subgroups of patient populations. An example is the substantial rate of non-responders in anti-TNF treatment in IBD [15]. Although this was not investigated in the current thesis, the analysis method that we applied (Random Forest) can also be used to identify microbial taxa that are able to predict treatment response and thereby may help future initiatives.

Clinicians, researchers and companies/industry are currently investigating the possibility to develop microbiota-based treatments. Although theoretically promising, evidence-based microbiota targeted therapeutics are still limited at this moment. Products containing beneficial microbes such as *Lactobacillus, Bifidobacterium* and *Saccharomyces* are currently available to combat travelers' or antibiotic-associated diarrhea. However, interventions for intestinal diseases are not readily available or implemented in standard health care, in part due to limited methodological quality of the studies and too generalized (non-targeted) approaches. Further insight in microbial perturbations and how microbiota profile changes contribute to disease development or progression, will aid in designing more targeted interventions. One of the few microbiota interventions available for intestinal disease is fecal transplantation, which 'replaces' the intestinal microbiota of diseased individuals with the fecal microbiota of a healthy individual. This method has been proven to be effective against (recurrent) *Clostridium difficile* infections, but potential risks (*e.g.* transfer of pathogens or antibiotic resistance genes) have to be taken into account. Safety issues in fecal transplantation, especially on long term, need to be addressed.

The bacterial taxa that we identified to be associated with diseases and disease states in this thesis are valuable in this respect and can also provide new insights regarding the role of the microbiota in disease. For example, *Bacteroides fragilis* (amongst others) was found to be associated with exacerbations in CD. A subgroup of *B. fragilis* is able to produce toxins which can affect intestinal permeability and aggravate intestinal inflammation. On the other hand, we also found *Faecalibacterium prausnitzii* (amongst others) to be associated with remission of CD. *F. prausnitzii* is known to be a major butyrate producer. Butyrate is a short chain fatty acid (SCFA) that is a major energy source for epithelial cells and plays an important role in maintaining the intestinal barrier integrity and has anti-inflammatory effects on the host [16]. *F. prausnitzii* is also able to dampen inflammation for example by producing metabolites that inhibits NFκB activation and IL-8 secretion [17]. Successful microbiota targeted interventions can act by promoting the growth of microbes associated with remission or combat microbes associated with active inflammation by using microbial compounds, antagonists and pre- or probiotics.

Perspectives

Although the concepts on the intestinal microbiota as non-invasive disease markers and on microbial intervention strategies are promising, the successful translation of such (basic) microbial research into clinical applications remains challenging. Therefore, further validation, standardization and tight collaborations between clinicians, scientists and industry is needed.

Although the microbiota obtained from intestinal biopsies are considered to be more valuable for examining the role of the microbiota in several disease states, due to the proximity of the microbiota to the host, these samples are not suitable for routine monitoring. Repeated samples are necessary and obtaining biopsies via endoscopy requires invasive and expensive procedures.

Feces or exhaled breath are rather easy to obtain and can give a valuable reflection of the microbiota composition and/or its activity. Immediate freezing at -80 °C or direct DNA isolation after defecation is the best method to investigate the microbiota composition, but this is often not possible when including patients from clinical or outpatient settings. The choice of sampling and storage methods is therefore crucial in the realization of reliable microbiota-based monitoring or diagnosing tools. In addition, costs for sampling and storage methods are a decisive factor in the realization of novel microbiota-based tools in the clinic. Various companies have developed patient friendly sampling and storage methods/kits that guarantees to keep the microbial integrity intact, even after several days at room temperature, making it possible to send the samples via mail. However, it is important that these methods/kits are affordable. Since we found that the fecal microbiota composition can be kept stable up to 24 hours at room temperature, auto-collection of fecal samples at the patients' home is possible in case the samples are delivered at the hospital or diagnostics department within 24 hours. This is especially convenient when the moment of sampling coincide with doctors appointments (as in the case of the IBD-South Limburg cohort). In contrast to feces, exhaled air is easy to collect

at any time and at any place in any patient. Previous studies from our group showed that analyses of volatile organic compounds (VOCs) in exhaled air resulted in sensitive and specific VOC profiles for the diagnosis of IBD, irritable bowel syndrome and liver cirrhosis [18–20]. In the current thesis, we found a correlation between VOCs and microbial taxa specific for disease status (remission or exacerbation). This may be valuable for elucidating the functionality of the microbiota and its role in Crohn's disease. Further studies are needed to investigate whether combining microbiota analyses and metabolites in exhaled air can increase their diagnostic accuracy as a disease (activity) marker.

Developing microbiota-based treatments often involves delivering specific strains, microbial products or other molecules to the intestine via the oral route. Targeted delivery of these compounds directly in the intestine, thereby reducing deleterious effects for *e.g.* gastric acid, bile and pancreatic enzymes, may increase the efficacy. Delivery platforms (*e.g.* enteric coatings) and synthetic biology can be helpful. The latter is a relatively new and upcoming branch of biology that involves designing or redesigning (biological) compounds and (biological) systems for useful purposes. Bacterial strains can be modified to result in an increased production of beneficial metabolites or may be engineered in such a way to have an advantage over other microbes (*e.g.* competing for nutrients or adhesion sites). Novel interventions need to be tested prior to implementation in the clinic. This is often done in animal models, but it is important to note the intrinsic differences between these and humans. *In vitro* intestinal models such as the TIM system can be of additional value to test delivery systems, since they closely mimic the human gastrointestinal tract [21,22].

In summary, this thesis contributes to the ongoing debate whether and how microbiota can be used as a diagnostic tool, marker for disease progression and predictor for response to therapeutic interventions. We have shown that for the development of microbiota-based markers, it is better to search for a set or combination of multiple members of the microbiota rather than to focus on only a few members. Metabolites produced by the microbiota are also able to reflect or even influence host' health and should be taken into account. In addition, we provided information for selecting the correct sampling and storage method applicable in the clinical setting, which is patient-friendly and maintains the microbiota composition. Microbiota research is fast-pacing and we are currently moving from describing the microbiota composition to elucidating their functions and dynamics. There is a need for mechanistic studies to elucidate interactions between members of the microbiota together and their host. This knowledge will enable the development of new interventions to manipulate the microbiota with the intention to improve human health. It should be taken into account that efforts to manipulate the interactions between the host and microbiota may result in unforeseen adverse outcomes (*e.g.* potential pathogenicity of commensal microbes). Therefore, further interdisciplinary investigations will lead to better understanding of the microbiota-host interactions and will contribute to safe microbiota-based interventions against a range of disorders.

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List of publications

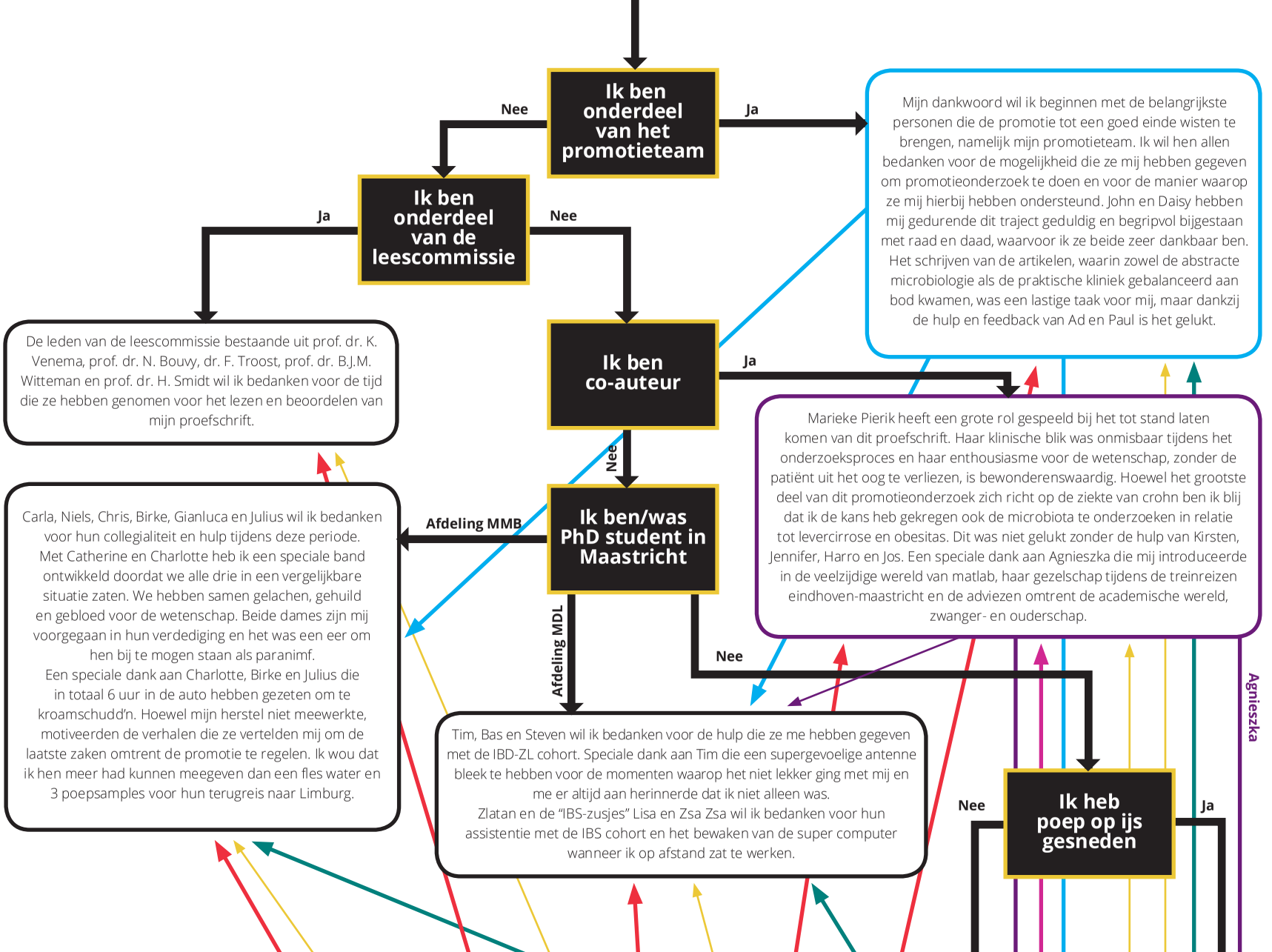
LIST OF PUBLICATIONS

Tedjo DI, Jonkers DMAE, Savelkoul PHM, Masclee AAM, van Best N, Pierik MJ, Penders J. The effect of sampling and storage on the fecal microbiota composition in healhy and diseased subjects. *PLoS One.* 2015;10(5):e0126685. doi:10.1371/journal.pone.0126685

Tedjo DI, Smolinska A, Savelkoul PHM, Masclee AAM, van Schooten FJ, Pierik MJ, Penders J. The fecal microbiota as a biomarker for disease activity in Crohn's disease. *Scientific Reports.*2016. doi:10.1038/srep35216

9

Dankwoord


Ik ben onderdeel van het promotieteam
Nee
Ja
Mijn dankwoord wil ik beginnen met de belangrijkste personen die de promotie tot een goed einde wisten te brengen, namelijk mijn promotieteam. Ik wil hen allen bedanken voor de mogelijkheid die ze mij hebben gegeven om promotieonderzoek te doen en voor de manier waarop ze mij hierbij hebben ondersteund. John en Daisy hebben mij gedurende dit traject geduldig en begripvol bijgestaan met raad en daad, waarvoor ik ze beide zeer dankbaar ben. Het schrijven van de artikelen, waarin zowel de abstracte microbiologie als de praktische kliniek gebalanceerd aan bod kwamen, was een lastige taak voor mij, maar dankzij de hulp en feedback van Ad en Paul is het gelukt.
Ik ben onderdeel van de leescommissie
Ja
Nee
De leden van de leescommissie bestaande uit prof. dr. K. Venema, prof. dr. N. Bouvy, dr. F. Troost, prof. dr. B.J.M. Witteman en prof. dr. H. Smidt wil ik bedanken voor de tijd die ze hebben genomen voor het lezen en beoordelen van mijn proefschrift.
Ik ben co-auteur
Ja
Nee
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Ik ben/was PhD student in Maastricht
Afdeling MMB
Afdeling MDL
Nee
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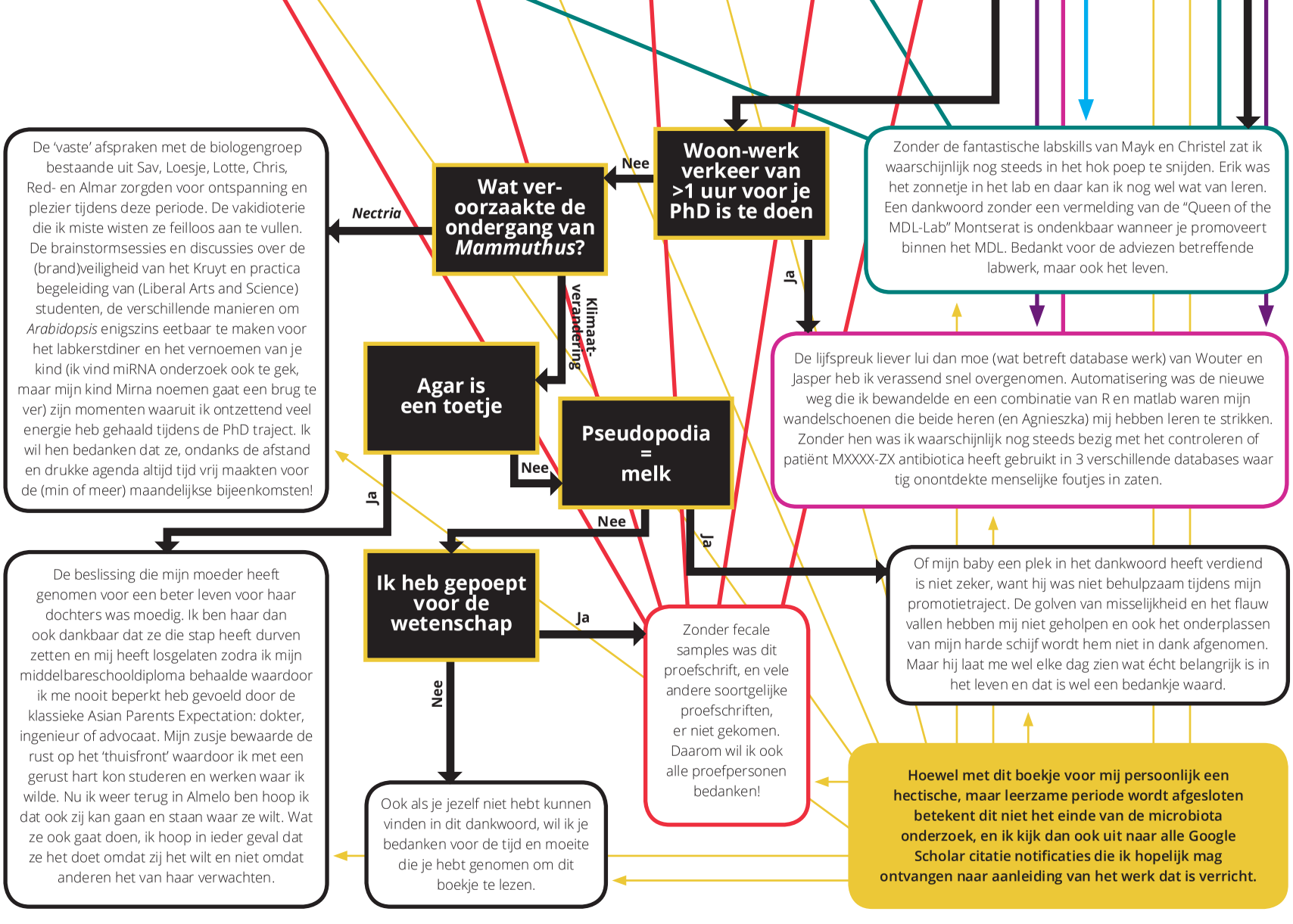
Ik heb poep op ijs gesneden
Nee
Ja
Agnieszka


Woon-werk verkeer van >1 uur voor je PhD is te doen
Nee
Ja
Wat ver-oorzaakte de ondergang van *Mammuthus*?
Nectria
Klimaat-verandering
Agar is een toetje
Nee
Pseudopodia = melk
Ja
Nee
Ja
Ik heb gepoept voor de wetenschap
Ja
Nee
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9

Curriculum Vitae

CURRICULUM VITAE

Danyta Isthylova Tedjo was born on the 7th of December 1988 in Surabaya, Indonesia. In 1996 she emigrated to Almelo, the Netherlands, where she finished her primary school and obtained her VWO Gymnasium degree from the Pius X college. In 2007 she attended Utrecht University where she obtained her bachelor diploma in Biology. Her thesis, written at the department plant-microbe interactions under supervision of dr. Peter Bakker, focused on the application of genomics, transcriptomics and proteotomics for entangling plant-microbe interactions in the rhizosphere. After completing her bachelor degree she continued her master's degree in Infection and Immunity at the University Medical Centre Utrecht. During her first internship she worked under the supervision of dr. Sergey Konstantinov on the functional characterization of hypothetical life-stock associated *Staphylococcus aureus* exoproteins. She worked under supervision of dr. Sylvia Brugman at the Wilhelmina Children's Hospital in Utrecht for her second internship, which consisted of two projects. The first project aimed to investigate the recruitment of different types of immune cells to the intestine of *Danio rerio* in response towards bacteria and bacterial products, while the second project focused on the establishment of *Danio rerio* intestinal organoids. Her master's thesis, written at the RIVM under supervision of dr. Vinay Saluja, was focused on the production and formulation of polymer-based vaccine delivery systems. After obtaining her master degree, she started as a PhD candidate at Maastricht University in 2012 at the departments medical microbiology and gastroenterology-hepatology. Her PhD project focused on the characterization of the microbiota in patients with gastrointestinal diseases, under the supervision of dr. Daisy Jonkers, dr. John Penders, prof. dr. Ad Masclee and prof. dr. Paul Savelkoul.