Diplomarbeit

Detecting the role of fungi, bacteria and archaea in intestinal biofilms of IBD and IBS patients

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Elisabeth Maria Orgler

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Ao. Univ.-Prof. Dr. Christoph Gasche

und

Maximilian Baumgartner, MSc.

unter der Betreuung von

Univ.-Prof.in Dr.in Christine Moissl-Eichinger

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Zusammenfassung

EINLEITUNG Die Inzidenz Chronisch Entzündlicher Darmerkrankungen (IBD) und Reizdarm-Syndroms (IBS) steigt kontinuierlich in der westlichen Welt, während die Ätiologie unbekannt bleibt. Vorhergegangene Studien legen eine multifaktorielle Pathogenese für beide Erkrankungen nahe, wobei das Mikrobiom eine zentrale Rolle in der Pathophysiologie einzunehmen scheint. Es wurden bereits charakteristische Zusammensetzungen des Mikrobioms, welche sich deutlich von Gesunden unterscheiden, für Colitis ulcerosa (UC), Morbus Crohn (CD) und IBS entdeckt. Es wird vermutet, dass Interaktionen von Mikroorganismen mit der intestinalen Mukosa teilweise den epithelialen Zellzusammenhalt schwächen und immunologische Vorgänge, welche zu Entzündung und Krankheitsentwicklung führen, begünstigen. In einer großen Koloskopie-Kohorte am Wiener Allgemeinen Krankenhaus wurden makroskopisch-sichtbare Biofilme im Darm von IBD- und IBS- Patientinnen/ -Patienten entdeckt. Die Hypothese dieser Studie lautet, dass Biofilme charakteristische Zusammensetzungen aus unterschiedlichen Mikroorganismen besitzen und die Pathophysiologie von IBD und IBS stark beeinflussen.

METHODEN Biofilmproben von UC-, IBD-Unclassified (IBD-U)-, IBS- und Kontroll-Patientinnen/ -Patienten wurden im Rahmen einer Koloskopie entnommen. Die DNA von Biofilm- und dazugehörigen Stuhlproben wurde extrahiert und Polymerase Kettenreaktion (PCR) für Fungi, Archaea und Bakterien wurde durchgeführt. Die Proben wurden mittels MiSeq Illumina sequenziert und die Ergebnisse analysiert, um signifikante Unterschiede in der mikrobiologischen Zusammensetzung von Biofilm- und Stuhlproben zu erkennen.

ERGEBNISSE Biofilme sind polymikrobielle Mikrolebensräume bestehend aus Fungi, Archaea und Bakterien und ähneln in ihrer Zusammensetzung, was Fungi und Archaea betrifft, ihren gematcheden Stuhlproben. Allerdings zeigten sich im Stuhlmikrobiom nur für Archaea signifikante Unterschiede zwischen Patientinnen/ Patienten mit und Patientinnen/ Patienten ohne Biofilme. Außerdem wurde ein krankheitsspezifisches Clustering bei UC und IBS für die bakterielle Zusammensetzung von Biofilmen entdeckt. Des Weiteren wurde ein Biofilm des Magens analysiert, welcher einen hohen Anteil an *Streptococcus* und *Lecanoromycetes* zeigte.

DISKUSSION Endoskopisch-sichtbare Biofilme im Colon sind eine medizinische Neuentdeckung. In dieser Studie konnten spezifische Veränderungen des Mikrobioms von Biofilm-positiven Patientinnen/ Patienten gezeigt werden, was Ausdruck einer Dysbiose sein kann. Die Ähnlichkeit eines Biofilms zu seiner gematcheden Stuhlprobe zeigt, dass Fungi und Archaea im Stuhl wahrscheinlich für die Entstehung von Biofilmen

mitverantwortlich sind. Des Weiteren konnte gezeigt werden, dass sich Biofilme je nach Krankheitstyp (IBD vs. IBS) unterscheiden, was auf unterscheidliche Pathomechanismen hinweist. Es könnte sich hierbei um die ersten bei IBS entdeckten makroskopischen Veränderungen handeln und Biofilme könnten dazu beitragen die Pathophysiologie von IBD und IBS besser zu verstehen. Allerdings braucht es weitere Studien, um die Rolle von Biofilmen im Gastrointestinalen Trakt und ihren Einfluss auf IBD und IBS zu evaluieren.

Abstract

INTRODUCTION Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS) have both rising incidences in Western societies, while etiologies remain unclear. Past studies have contributed to increasing evidence of multifactorial pathogeneses for both diseases, with the microbiome as a central role in disease pathophysiology. Microbial signatures for Ulcerative Colitis (UC), Crohn's Disease (CD) and IBS, differing significantly from healthy controls, have been detected. Microbial interactions occurring at the intestinal mucosa, partly impairing epithelial barrier function, are thought to be closely involved in immunologic mechanisms leading to inflammation and disease development. During colonoscopies of a large patient cohort at the Vienna General Hospital, macroscopically visible biofilms were detected in the intestines of IBD and IBS patients. We hypothesize that these biofilms harbor disease-specific polymicrobial signatures and substantially influence disease pathogenesis.

METHODS Biofilm samples of UC, IBD-Unclassified (IBD-U) and IBS patients were collected during colonoscopy. DNA of Biofilm and matched stool samples was extracted and polymerase chain reaction (PCR) was performed for fungi, archaea and bacteria. Sequencing with MiSeq Ilumina was conducted and reads were analyzed to detect significant differences in microbial composition of biofilm and stool samples.

RESULTS Biofilms are polymicrobial microenvironments consisting of fungi, archaea and bacteria, and biofilm composition resembles the microbiome of matching stool samples concerning fungi and archaea. However, fecal mircrobiomes of patients with and patients without biofilms only show significant differences in archaeal composition. Significant biofilm compositions for disease phenotypes could only be detected when studying bacteria, not for fungi and archaea. Finally yet importantly, a gastric biofilm was studied and revealed high abundance of *Streptococcus* and *Lecanoromycetes.*

DISCUSSION Endoscopically visible biofilms of the colon are a completely novel finding. In this study, we showed specific shifts in the microbiome of biofilm-positive patients, pointing at gut dysbiosis. The resemblance of biofilms to their matched stool samples shows, that fungi and archaea might be responsible for the development of biofilms. Furthermore, we showed that biofilm composition depends on disease phenotype (IBD vs. IBS), indicating different pathomechanisms. Biofilms might be the first macroscopic alterations detected in IBS and could help to better understand disease pathophysiology of IBD and IBS. However, further studies are needed to evaluate the role of biofilms in the gastrointestinal tract and their impact on IBD and IBS.

Glossar und Abkürzungen

IBD	Inflammatory Bowel Disease
CD	Crohn's Disease
UC	Ulcerative Colitis
CRC	Colorectal cancer
IBD-U	Inflammtory Bowel Disease, Unclassified
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
IL	Interleukin
T_H	T helper cell
TNF	tumor necrosis factor
Ig	Immunoglobulin
PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptor
INF γ	Interferon γ
IBS	Irritable Bowel Syndrome
IBS-D	Irritable Bowel Syndrome, diarrhea subtype
IBS-C	Irritable Bowel Syndrome, constipation subtype
IBS-M	Irritable Bowel Syndrome, mixed symptoms subtype
PI-IBS	post-infectious IBS
ENS	enteral nervous system
BA	bile acids
BS	bile salts
PCoA	principal coordinate analysis
SCFA	short chain fatty acids
5-HT	serotonin
ST	stool sample
BFF	biofilm flush
PCR	polymerase chain reaction
ITS	Internal Transcribed Spacer
ASV	amplicon sequence variant
LEfSe	Linear discriminant analysis Effect Size
ST-BF-pos	stool samples of biofilm-positive patients
ST-BF-neg	stool samples of biofilm-negative patients

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1 Introduction

1.1 Inflammatory Bowel Disease

Inflammatory Bowel Diseases (IBD) comprise two major forms of chronic inflammation of the gastrointestinal tract: Crohn's Disease (CD) and Ulcerative Colitis (UC) (1). In both diseases, the intestinal mucus layer is damaged by an excessive inflammatory response likely triggered by dysbiosis (2). The destruction of epithelium leads to inflammation, which might include bleeding and ulceration. IBD occurs in a chronic relapsing manner, with remittent or progressive course (1). Due to oxidative damage and inappropriate repair following the chronic inflammation, IBD is accompanied by an increased risk for developing colorectal cancer (CRC) (3,4).

The onset of IBD typically takes place during young adulthood and symptoms include frequent (bloody) diarrhea, abdominal pain and weight loss. Life quality is impaired, work ability decreases and the financial burden for health-care systems is substantial (5).

Etiology remains unresolved, however increasing evidence suggests four components to disease development: genetic predisposition, environmental factors, microbiome alterations and as a consequence to these, immune response. World-wide incidence of IBD is increasing, with a prevalence of 0.3 % in Western countries (6).

Though CD and UC share some disease patterns and symptoms, there are important differences to note, as both forms of IBD demand specific treatments (7).

UC primarily affects the colon, ascending from the rectum and continuously spreading orally, typically not surpassing the ileocecal valve. CD however can appear anywhere in the gastrointestinal tract, spreading discontinuously and in a patchy manner, often referred to as skip lesions. Common locations for CD are the terminal ileum and colon, but also upper parts auf the small bowel or esophagus and stomach. Stenosis and fistulas, especially in the perianal region, are a specific feature in CD (8).

UC only affects the mucosa and submucosa of the gut, CD has a transmural pattern, affecting all tissue layers. Histologically, CD shows a dense infiltration of lymphocytes and macrophages, sometimes also non-necrotizing granulomas, whereas in UC an invasion of neutrophils may be predominant (1,8).

Symptoms may vary in both UC and CD. Typically, in UC diarrhea occurs with a high frequency, extending to up to 15 defecations per day and typically bloody, whereas in CD diarrhea may be less severe and rarely involves visible blood. Abdominal pain often reaches

a maximum before and during defecation in UC, declining afterwards. In CD, pain often persists throughout the day and is commonly located in the right lower abdomen, typically after meals (9).
Extra-intestinal symptoms are common in CD patients, including inflammation of joints, skin and eyes. In UC on the other hand, there is a subgroup of patients with primary sclerosing cholangitis (10).

Sometimes gastrointestinal appearance may apply to both UC and CD diagnostic criteria, complicating diagnosis For instance, inflammatory areas might only occur in the colon therefore indicating UC, but the type of mucosal lesions might resemble CD more accurately; or typical macro- and microscopic features of both diseases might be present in different locations of the gastrointestinal tract. When this occurs and differentiation between UC and CD is difficult, patients may be diagnosed with IBD-U, i.e. IBD, unclassified, orpreviously called indeterminate colitis (1,8).

Therapy of both IBD forms varies depending on type, disease course and individual patient. For acute relapse, glucocorticoids are best to reduce inflammation and symptoms. Long-term therapy for UC focuses on the use of mesalazine, also called 5-aminosalicylic acid. For CD the safe and inexpensive mesalazine is not considered effective. Instead, immunotherapy, including azathioprin and biologicals like infliximab and adalimumab, is used (7–9).

1.1.1 Epidemiology

1.1.1.1 Established Populations

Countries with an already established high prevalence of IBD include North America, Europe, Australia and New Zealand. Disease increment took place in the second half of the twentieth century. Examples of extreme increase of IBD include one study from Olmsted County, MN, USA, where UC incidence rose considerably from 0.6 to 8.3 per 100,000 person years between the 1940s and the 1980/90s (11). Since the 1990s incidence is reported somewhat stable in westernized countries, with varying estimates . Today incidence for UC ranges from 8.8 – 23.14 to 0.97 – 57.9 per 100,000 person-years in North America and Europe, respectively. For CD incidence lies between 6.3 – 23.82 and 0.0 – 15.4 per 100,000 person-years in North America and Europe, respectively (6).

Prevalence remains somewhat stable as well, for UC with 0.14 – 0.29 % and 0.002 – 0.51 % and for CD with 0.96 – 0.32 % and 0.002 – 0.32 % in North America and in Europe, respectively. According to this estimate about 1.5 million people in North America and 2.5 million in Europe suffer from IBD altogether (6).

Disease trends include a higher risk for urban than rural populations, and in Europe a historic North-South gradient, with high rates in some Scandinavian countries, that has been replaced by a West-East gradient after the fall of the iron curtain. Incidence is not affected by gender, but by ethnicity. Three-fold higher risk in Ashkanasi Jews, than non-Jewish populations and higher incidences in Caucasians than non-Caucasian, are examples (11).

1.1.1.2 Emerging Populations

Traditionally exhibiting lower IBD rates, countries in Asia, Africa and South America, are registering rapidly increasing incidence rates. Most affected are newly industrialized countries, suggesting important risk factors coming along with Western lifestyle (11).

Incidence in Asian, South American and African countries amount to 0.15 - 6.76 and 0.06 - 8.4 per 100,000 person-years for UC and CD, respectively. Prevalence of UC and CD lies around 0.005 - 0.1 % and 0.001 - 0.05 %, respectively (6,12).

The epidemiologic data suggests that disease rates remain lower than in Western countries. However, the annual percentage change of the incidence rates are steadily increasing, showing a trend similar to the ones in Western countries during the second half of the twentieth century (6).

Interestingly, the incidence of UC in Indian migrants living in the UK approximated itself towards the one of the UK population. This risk increase was present in both first- and second-generation immigrants, but strongest in the latter. However, this was not found for CD. In most population studies, CD incidence seems to increase only a decade after UC already has (11).

1.1.2 Etiology

The etiology of IBD remains unresolved, as disease pathomechanisms is not yet fully understood (1,13). However, evidence points towards a pathogenesis with multiple factors involved, which when coinciding lead to IBD. A correlation of genetic susceptibility, environmental risk factors, microbiome composition and host immune response as a function of the first three factors is thought to account for disease development (14). Environmental factors seem to contribute greatly, explaining the increasing prevalence of IBD in the last

decades, and in shaping the structure of the gut microbiome. Today IBD pathophysiology is understood as a multifactorial disease of the intestinal barrier function, originating from an abnormal immune response to the microbiota residing in the intestine (7,13–15).

1.1.2.1 Genetic susceptibility

Initially, a genetic component was suggested, when twin and familiar aggregation studies were conducted in IBD-positive populations. A strong hereditary factor was observed, with a positive family history for IBD in 2 - 14 % and 8 - 14 % of CD and UC patients, respectively (11). The risk of developing IBD with a positive first-grade relative family history amounts to 5 - 8 % and 1.6 - 5.2 % for CD and UC, respectively. Concordance rates for monozygotic twins even reach 20 - 50 % and 16 % for CD and UC, respectively (11).
This strong genetic risk factor is reflected in a multitude of recognized genes associated with susceptibility to IBD development. Over 200 genetic risk loci have been identified for IBD so far, of which most are shared between UC and CD (16). Additionally, 50 % of IBD risk loci are also associated with other autoimmune or inflammatory diseases (15). However, to be susceptible to disease development, more than just one risk locus has to be present. Also, genetic susceptibility seems to play a bigger role in CD, making environmental risk factors even more relevant in UC (14–16).
Genes involved in IBD pathogenesis include immune regulation, epithelial barrier function, mucus production, microbial defense, reactive oxygen species generation and cellular homeostasis (11,13–15).
The intracellular pattern recognition receptor nucleotide-binding oligomerization domain-containing protein 2 (NOD2) was the first identified IBD gene and is expressed in dendritic cells, macrophages, Paneth and intestinal cells. Individuals with one of the three identified gene variations of NOD2, carry an up to 40-fold increased risk of developing CD (14). NOD2 is crucial for the production of α-defensin, an antimicrobial protein produced in the intestinal epithelium by Paneth cells. Defensins play an important role in innate immunity and are involved in protecting the epithelium from microbial invasion (17). In addition, NOD2 seems to be involved in the stimulation and regulation of toll-like receptor signaling, which allows the innate immune system to recognize microbes. Thus, deficiency of NOD 2 leads to attenuated recognition and response to microbial invasion (14,15).
Also involved in microbial recognition and defense is the Interleukin (IL) 23 - T helper cell (T_H) 17-pathway, which includes multiple gene loci associated with IBD risk. However, the majority of genetic loci (70 %) are non-coding variants and their roles remain unclear (2).

1.1.2.2 Environmental Triggers

As mentioned before, environmental factors are gaining increasing attention, due to rising incidence and a big part of etiology not being explained by genetics (11). Many of the following factors are due to industrialization, which could account for the emerging incidence of IBD in newly westernized countries. By modulating the microbiome and host factors (as mucosal barrier and immune response), environmental triggers might influence disease risk and development in the genetically susceptible individual. The wide range of environmental factors includes smoking, diet, antibiotics, hygiene, psychologic state, location, early life and breastfeeding, to mention only a few (11,18).

The use of antibiotics, especially in early childhood has been associated with IBD diagnosis repeatedly. Antibiotics have a persistent effect, as one study has shown, a five-day medication with ciprofloxacin shifts the microbial composition significantly and some bacterial taxa do not recover within six months (19). A Canadian study demonstrated that the use of antibiotics within the first year of life might play a crucial role in IBD development. 58 % of IBD patients had received at least one antibiotic treatment early in life, compared to 39 % of healthy controls (20). In addition, a meta-analysis of eleven studies concluded that all antibiotics except penicillin are associated with an elevated risk for IBD diagnosis (21).

Smoking is one of the most puzzling risk factors in IBD. Since 1982 it is known, that the otherwise harmful addiction has a protective effect on UC (22). However, smoking acts as a risk factor for the related CD (23). Many studies have tried to explain the difference in disease effect, however none have ultimately succeeded to explain the pathomechanism. Hypothesizes include effects of smoking on the gut mucus production and barrier, oxidative stress and smooth muscle tone (24).

Diet plays an important role as an environmental risk factor. The microbiome is strongly influenced by nutrition and the microbial composition affects IBD risk. Dysbiosis has been associated with both UC and CD and can lead to abnormal immune responses in the gut mucosa, perhaps consequently leading to IBD (18). A large prospective cohort study has shown that women with high fiber intake have a reduced risk to develop CD, whereas those women with little fiber intake are more likely to be affected by CD (25). Dietary intake of

omega-3 fatty acid has been associated with a decreased risk of UC, whereas the intake of omega-6 fatty acid might increase the risk. One mechanism, by which omega-3 fatty acids could decrease UC risk, is its ability to suppress tumor necrosis factor (TNF) production by T_H1 cells (25). Interestingly, some micronutrients also affect disease risk. For example, a high intake of zinc has been shown to decrease CD risk in women. Low zinc levels in CD patients is associated with worse outcome and by normalizing zinc levels, outcomes are improved. Also, high vitamin D levels are associated with decreased CD risk (18). Contributing to modern lifestyle risk factors, emulsifiers have been found to alter microbial composition towards a pro-inflammatory state. This study was conducted in mice with two relatively common emulsifiers, carboxymethyly cellulose and polysorbate-80. These results point towards inadequate food safety, especially in the field of food additives.In general, food additives are screened for adverse effects in healthy animals, thereby neglecting the effect they might have on susceptible persons (as IBD patients with an instable microbiome and reduced mucosal barrier) (26). In a Guidance Report the International Organization for Inflammatory Bowel Diseases has recently developed specific recommendations on nutrition for IBD patients based on findings of clinical and basic research. This includes the recommendation to reduce consumption of artificial sweetners, emulsifiers, carrageenans, nanoparticles as titanium dioxide, trans-fats, and unpasteurized dairy products (risk of infection). In addition, UC patients are advised to reduce red and processed meat, as well as myristic acids (palm oil, coconut oil and dairy), whereas CD patients may be advised to increase the intake of fruits and vegetables (27).

Environmental air pollution could also attribute to IBD. Studies have shown that acute exposure to airborne particle matter can cause increased permeability of the gut mucosa, whereas chronic exposure alters the gut microbiome and exaggerates pro-inflammatory cytokine production. However, there have been no conclusive findings in epidemiologic studies on how air pollution affects IBD development (18).

The microbiome is an ever-changing system, however critical developmental steps take place during the first year of life, making it susceptible to disturbances (18). Birth mode and breastfeeding have immense influences on the composition of the microbiome. Cesarean delivered babies tend to have a less diverse microbiome, and breastfeeding shows a durable and favorable effect on the microbiota. Though these are risk factors for a less diverse microbiome, no direct risk effects on IBD were shown yet (18).

1.1.2.3 Microbiota

The microbiome as a pathophysiological factor in IBD pathogenesis will be discussed in 1.3.3.

1.1.2.4 Immune Response

As many of the genetic susceptibility loci for IBD are linked to recognition and response to microbes, it becomes clear that the dysregulation of the gut immune system has a central role in pathogenesis. One hypothesis proposes a pathogenic response of the immune system against commensal microbial flora, therefor overreacting and causing inflammation (28). Supporting this hypothesis, T cell responses and Immunoglobulin (Ig) G secretion targeted at commensal bacteria is a common feature in IBD (28).

One mechanism of impaired response to microbes, concerns the recognition of pathogen-associated molecular patterns (PAMPs). PAMPs are small molecules of pathogenic bacteria, often located in the cell membrane. Pattern recognition receptors (PRRs), including toll-like receptors on dendritic cells, sense PAMPs, recognize the pathogenic bacterium and activate an immune response. Commensal gut bacteria carry PAMPs closely resembling pathogenic microbes, which however are not identified as harmful and therefore tolerated. Even small genetic defects in PAMP recognition could affect the correct execution of this task and lead to a continuous immune response to commensal flora, leading to chronic inflammation (2,29).

Another phenomenon regards the properties of commensal bacteria. Bacterial proteases might play an important role in inducing disease, by impairing the gut epithelium. The commensal *Enterococcus faecalis* possesses a zinc-dependent metalloproteinase (gelatinase) which targets intercellular junction complexes, like E-cadherin and occluding. By disrupting the integrity of the mucosa, invasion and inflammation might be triggered. However inflammation only occurs in individuals with genetic susceptibility, stressing the interplay of genetic predisposition, microbial factors and immune response (2,30).

Supporting the importance of the immune system in IBD, antibodies against fungi or with autoimmune potential are enhancing findings. Up to 63 % of CD patients test positive for anti- Saccharomyces cerevisiae antibodies, with evidence that half of these patients do not have the antibodies before disease onset (31,32). In contrast, anti-neutrophil cytoplasmic antibodies with perinuclear staining can be a marker for UC, with 58 % being positive (32).

Both forms of IBD are sustained through complex interplays of the innate and adaptive immune system, with different pathways being activated in CD and UC. CD pathogenesis is thought to evolve around T_H1 - cell response, where due to high IL-12 levels naïve T cells differentiate into T_H1 cells (14,33). These T_H1 cells start producing Interferon γ (INF-γ) and TNF-α, which initiates a cascade leading to mucosal inflammation (13). Another T_H form, T_H17, is central to UC and CD. T_H17 cells produce pro-inflammatory cytokines including IL-17 and TNF-α, while their own differentiation is activated by IL-23. IL-23 is produced by phagocytic, endothelial and dendritic cells, when PRR binding occurs (14,33). As stated before, the recognition of PAMPs from commensal bacteria residing in the gut, is exaggerated in individuals with IBD susceptibility. This could lead to chronically high IL-23 levels causing activation of T_H17 cells and consequently inflammation. T_H17 cells probably act as a central part in inflammation and IBD pathogenesis, but disease pathomechanism is not yet completely understood (2,7,13,33).

1.2 Irritable Bowel Syndrome

Irritable Bowel Syndrome (IBS) is a multifactorial, functional gastrointestinal disease with a broad spectrum of symptoms. Prevalence is high, especially in the Western world and IBS counts to one of the most frequent causes for gastroenterological consultations. Etiology remains unknown, with evidence suggesting disturbances in the brain-gut axis, dysbiosis, low grade inflammation and pathophysiologic changes in epithelial barrier function (34).

Common symptoms of IBS include abdominal pain, bloating, abdominal distension, diarrhea and constipation, without any structural correlate found in diagnostic tests. Symptoms can vary inter- and intrapersonally, sometimes leading to work inability and high reduction of quality of life (34).

IBS can be divided in three subtypes, according to bowel habits. Individuals with diarrhea as prominent symptom are classified as IBS-D, whereas IBS-C stands for constipation as the predominant symptom. Mixed or fluctuating bowel movements, with alternating diarrhea and constipation are classified as IBS-M (35).

IBS is usually a diagnosis of exclusion and diagnosis can be very challenging for clinicians, as symptoms may imitate IBD, other severe diseases or another subform of functional bowel disorders. The current definition of IBS is specified in the Rome IV criteria, which was published in 2016 (35). Central to IBS diagnostic criteria is recurrent abdominal pain, which must be present at least once a week on average, in the last three months. At least two of the following, other symptoms must be present: the pain is associated with a change in stool

frequency, the pain is related with defecation and stool consistency is altered. Symptoms must be present in the last three months and onset has to be at least six months before diagnosis. However, the diagnostic criteria for IBS have been altered contiuously over the years, making it a changing disease entity. For instance, recurrent pain has not been a diagnostic requirement before, now excluding many before diagnosed patients, for whom symptoms like altered stool frequency or bloating are most severe. This recurrent alteration of diagnostic criteria impedes consistency in diagnosis and recommened treatments. Furthermore, it hints at IBS being an umbrella term for several different diseases, which have not yet been determined. Future studies may help detect more specific diagnostic criteria and diseases subtypes (35).

1.2.1 Epidemiology

Measuring the prevalence of IBS poses many challenges, since diagnostic criteria depend mostly on not objectively measurable symptoms and the communication between doctor and patient. Also, epidemiologic studies often use different methods to detect IBS patients and different diagnostic criteria, which change every couple of years (35,36).

A large meta-analysis of international studies found the pooled prevalence of IBS to be 11.2 %, however data fluctuates between 1.1 % and 45 %, depending on region and diagnostic criteria used (e.g. Rome III vs. Manning criteria). Globally, the highest prevalence existed in South America with 21 %, whereas the lowest prevalence was found in Southeast Asia with 7 %. Most European countries, North America and China report a prevalence between 5-10 %, which makes IBS a frequent disease (36).

Risk factors mainly depend on age and gender, with females and individuals under the age of 50 years being at highest risk.(36) Women being more likely to develop IBS, might be explained by a different health understanding, more doctoral consultations and hormonal functions, as bowel movements are influenced by female hormones (34).

The developing of IBS after infectious gastroenteritis, poses interesting questions about etiology and pathogenesis. Post-infectious IBS (PI-IBS) occurs in up to 10 % of cases of gastrointestinal infections from bacteria, viruses or parasites. This evidence suggest that alterations in the microbiome, due to infections or antibiotic treatments while gastroenteritis is active, could be possible triggers of pathogenesis (34).

Interestingly, some other diseases are tightly associated with IBS. A significant overlap of about 20 % of IBS with other diseases, including chronic pelvic pain, reflux, dyspepsia or incontinence, is documented. In addition, other diseases are associated with IBS, among

them vulvodynia, chronic fatigue syndrome, sexual dysfunction and migraines. Also, psychiatric diseases, like anxiety disorder, somatization and depression, are associated with IBS. These evidences might point at involved factors for shared disease pathways, which still remain unknown (34).

1.2.2 Etiology

Recent studies provide increasing evidence for a multifactorial pathogenesis in IBS, however etiology remains incompletely understood. A combination of many factors is probable to cause IBS, including four main components. First, alterations in the gut mucosa physiology, with certain changes in the epithelial barrier and immune reactions, are likely to contribute. Secondly, the microbiome and structural abnormalities might play a central role in IBS pathogenesis. Third, the nervous system, including the brain and enteral nervous system (ENS) seem to have altered activation. Finally yet importantly, a genetic component with increased susceptibility has been suggested (34).

1.2.2.1 Alterations of the mucosa

Similarly to IBD, the gut mucosa seems to be altered in IBS, with an increased permeability for macromolecules, which usually cannot surpass the intestinal epithelium. Consequently, bacterial PAMPs might activate immune cells and trigger low-grade inflammation. Especially in IBS-D, but also post-infectious IBS, permeability is significantly increased compared to healthy controls (37). The responsible intercellular connections might be tight junctions, since one of the structural proteins, zonula occludens protein 1, is downregulated in IBS patients (38,39). This downregulation is associated with lower transepithelial resistance and increased permeability. Interestingly, by applying cleared supernatant from IBS biopsies, permeability is increased in cell lines. This suggests that intestinal permeability is triggered by a fecal soluble factor (39).

In another study, fecal supernatants from IBS-D patients were applied on mice and gut permeability was thereby increased compared to mice who received fecal supernatants from controls. Fecal serine protease was measured in all supernatant and found to be increased three-fold in IBS-D compared to IBS-C. This high level of fecal serine protease was associated with a slower redistribution of zonula occludens protein 1. The effects on permeability were shown to be preventable by serine protease inhibitors (38).

In addition, low-grade inflammation and mast cell activation seem to be a feature in many IBS patients. In the small and large intestines, T cells and macrophages are more abundant

in IBS patients compared to healthy controls.(40) Also, host-microbial interactions might be altered and systemic immune activation might occur in IBS, which is enforced by evidence of antibodies against gut bacteria and elevated levels of cytokines in peripheral blood (41).

1.2.2.2 Microbiome

The microbiome as a pathophysiological factor in IBS pathogenesis will be discussed in 0.

1.2.2.3 Nervous system

The nervous system might also be involved in developing and sustaining IBS, specifically the ENS and the brain.

As discussed before, IBS patients have bowel hypersensitivity, meaning recognizing a stimulus as painful, when healthy individuals would not do so. This might be due to an increase in sensory nerve fibers in the gut epithelium, and higher numbers of mast cells and lymphocytes in the gut mucosa of IBS patients. By emitting tryptase and histamine, mast cells stimulate and excite sensory nerves in IBS patients, therefor causing painful sensation. Also, proteases of mast cells, bacteria and the pancreas might contribute to higher activation of sensory nerves (42). The chronic stimulation with these mediators might alter the structure of the nerves, sensitizing them and leading them to overreact to stimuli. This mechanism is due to neural plasticity and could be key to chronicity of symptoms (34,37).

The brain gut axis might play a central role to IBS development as well. Through the autonomic nervous system, the brain influences gut motility, permeability, secretion of fluids and the activity of the immune system. In return, the condition of the intestine influences the brain's function and structure. An example of how closely intertwined the gut and brain are, was shown in a study checking for attitude towards symptoms and pain severity. Patients with bad coping strategies such as catastrophizing had significantly worse bowel symptoms, making cognitive behavioral therapy an important part of treatment. A disease model for IBS spanning pathophysiologic mechanisms as high attention to interoceptive signals (such as bowel movements, pain or bloating), the processing of these symptoms, their relation to emotional arousal and the expectation and experience of following gut symptoms has been suggested. All of these factors might be of importance to IBS development and especially sustainment (34,37,43).

1.2.2.4 Genetic susceptibility

Studies investigating genetic susceptibility to IBS have not been very successful so far. There have been several associations with certain genes, however many do not prove to be replicable (44).

Some studies have found variants or polymorphisms in genes coding for epithelial barrier function and immune modulators in IBS patients, which could explain some of the pathogenesis (44).

Others have found mutations in genes involved in the serotonergic system, which could be partly responsible for the altered brain response to visceral pain (34).

Interestingly, genes involved in bile acid (BA) production and responding receptors seem to be altered in many IBS patients, leading to an increase in synthesis. This is associated with faster colonic transit and might be common in IBS-D. A high availability of bile salts (BS) on the other hand is associated with the formation of bacterial biofilms, which is a key structure examined in our study and which will be discussed later in more detail (45,46).

To conclude, the etiology of IBS remains unknown. However, pathogenesis seems to be complex and multifactorial, whereas genetic susceptibility might be a minor risk factor (34).

1.3 Microbiome

The importance of the microbiome and its many effects on health and disease has become acknowledged in the last couple of years and therefore great interest in microbiome research has been sparked. Emerging evidence points towards a close relation between the microbiota and IBD or IBS and alterations in the microbiome are hypothesized to be triggers for both diseases (2,37).

1.3.1 Definition

The microbiome is defined as the total genetic information derived from all microbes present in and on the body. It encompasses approximately 3,8 x 10^{13} microbes, the majority of those residing in the gastrointestinal tract (47). Consisting of many different bacteria, fungi, archaea and viruses, the microbiome has a very diverse structure and the interactions between not only various species, but also many human cell types are complex. In fact, the microbial composition varies not only greatly between two individuals, but also over time intrapersonally and depends on one's health condition (48).

In addition, understanding causative relations in microbiome studies is challenging, however first studies using fecal matter transplantation in germ free mice establish a causal relationships between microbiome and disease pathogenesis (49,50).

The most abundant group of microorganisms is bacteria. Some estimates predict that 99 % of all microbial genes in the human microbiome stem from this group of prokaryotes. The bacterial composition is shaped by various factors, such as diet, genetics, drug intake, age, and geographics, making it difficult to describe an universal healthy microbiome (26). However, some bacterial strains seem to be shared by almost all humans and make up a core microbiome. It is clear that the phyla Firmicutes, Bacteroidetes, Proteobacteria and Actinabacteria are most abundant in the gastrointestinal tract. Furthermore, microbiomes with a high diversity in their bacterial composition are associated with a healthy state and tend to be more stable towards outer disturbances (26).

Another form of prokaryotes, archaea, are probable to be the second most abundant group of microorganisms present, thought to range around 1 % (51). However this approximation might underestimate the actual abundance, since most data comes from studies using bacteria-optimized DNA extraction and detection methods, whereas archaea require different extraction protocols and primers (51). It is clear so far that especially methane-producing archaea are most dominant in the gastrointestinal tract, such as *Methanobrevibacter smithii*, which is present in up to 96 % of human study populations. Although methanoarchaea have been shown to impact well-being and health condition, further studies to understand the role of the archaeome in disease context are lacking (51).

Fungi make up another component of the microbiome. Theses eukaryotes are less abundant, making up only about 0.03 – 0.1 % of all genetic material (52). However, their role might be underestimated, also due to methods being optimized for detecting bacteria. For detecting fungi, different and more complex extraction methods are required, in order to break their resistant cell wall. In addition, microbiome pipelines need adaptions for detection of eukaryotic genes (53). Another reason, fungi might be underestimated is because of their size – fungal cells are up to ten times bigger than bacteria, therefor could take up more space per cell in the gastrointestinal tract. This could mean that fungi have a bigger area of acting on the mucosa than often thought, when only interpreting the genetic data (54,55).

Compared to bacteria, fungal diversity is low, with yeast being dominant in the gastrointestinal microbiome. The most abundant fungal genera are *Candida, Malassezia* and *Saccharomyces*, with *C. albicans* being present in 80.8 %, *M. restricta* in 88.3 % and *S. cerevisiae* in 96.8 % of the Human Microbiome Cohort. The mycobiome seems, other than the bacterial component of the microbiome, not to be as stable. An individual core mycobiome has not been found yet (56).

Finally, yet importantly, the microbiome also harbors viral DNA. The virome is probably the least studied component of the microbiome. The majority of viruses present in the gastrointestinal tract are phages, which infect bacteria and transmit their DNA to them. To detect viral DNA is very challenging, since viruses, other than bacteria and fungi, do not have any universal genes shared among all viruses, but are all comprised of novel DNA sequences (53). Recent advances in sequencing technology led to a shift towards unbiased metagenomic sequencing, which promises novel discoveries how the virome shapes health and disease.It has been shown recently, that other than bacterial composition, the virome is unique to one person and the viromes of related family members do not resemble each other more than those of strangers. Therefor the viral composition seems not to be determined by the host's genes. Interestingly, although interpersonal diversity is high, the virome of an individual remains extremely stable, with 95 % of viruses remaining present over the course of a year (57).

1.3.2 Role in Health and Disease

The microbiome with all its components, bacteria, archaea, fungi and viruses, has an immense impact on human health and disease. By degrading and fermenting carbohydrates and fibers, the gut microbiota is involved in the digestive process and interacts closely with the gut epithelium, affecting energy uptake and storage. Through various mediators, the microbiota also communicates with intestinal epithelial and immune cells, influencing cell maturation and activity of the immune system (58).
The healthy microbiome is variable, altering between healthy individuals and changing over time intrapersonally. There are many factors contributing to this plasticity of the microbiome, some of these being environmental, others specific to an individual. Age is one factor accounting for the microbiome structure of an individual. In the first three years of life, the microbiome shifts greatly due to nutritional adaptions, but also because of ecological succession of different bacterial species (48).

Also, genetics play an important role in shaping the microbial community. However, environmental factors seem to be of greater importance. Encompassing diet, medication, geographic location, physical activity and many cultural traditions, environmental factors and lifestyle seem to be highly relevant in influencing the microbiome (48,59).

Due to the high individuality in microbiomes of healthy subjects, defining a universal healthy state remains challenging. A sign for a healthy condition however, seems to lie in the stability and resistance to stressors of the microbiome. This implements, that a healthy microbiome resists to altering its microbial composition when outer stressors occur or returns quickly to its initial state (48,60).

In addition, high bacterial diversity is a sign for a healthy microbiome. This is usually described with α diversity, which measures the richness and eveness of a given microbial community. One of the most prominent diversity parameters is the Shannon index, which is calculated by using the total number of species and the relative abundance of separate species, thereby picturing how rich and evenly bacterial species are spread in an individual (61).

Defining a microbiome as healthy by looking for specific bacterial species is too simplistic for the complex and diverse microbial system. Another form of describing a healthy microbiome has been suggested, determining a healthy microbiome by biological pathways and genes being present, rather than taxonomy. This emphasizes the importance of different microbial functions being carried out by the microbiome, instead of the presence of specific microorganisms. Since different species share genes and similar pathways, this hypothesis is promising and might explain why interpersonal diversity in healthy individuals is high (62).

Dysbiosis, a term to describe an imbalanced or unstable gut microbiome, is a common finding in various diseases. However, the extent and structural alterations of dysbiosis vary not only between diseases, but also between clinical studies, challenging efforts to find universal conclusions (63).

A meta-analysis combining 28 microbiome studies of different diseases published in 2017 by *Duvallet et al.,* found striking similarities in microbial dysbiosis patterns across diseases. Although there are disease-specific microbiome compositions, at least half of the associations apply to more than one disease, pointing towards shared responses of the microbiome in health and disease. For example, multiple genera of Firmicutes and Bacteroidetes seem to show a non-specific response to disease states. On the other hand, the order of Clostridiales is associated with health across studies. However, disease-specific

microbiome shifts do exist as well. Interestingly, some disease responses are distinguished by the presence of specific bacteria that might be pathogenic. Others on the other hand are characterized by the absence of health-related microorganisms (63).

1.3.3 Microbiome in IBD

The microbiome plays a crucial role in IBD pathogenesis and pathophysiology. As mentioned before, many environmental factors might act indirectly by influencing the microbial composition, which interacts closely with the gut epithelium and immune system of the host. By changing the gut microbiome towards a pro-inflammatory state, gut homeostasis might be disrupted and in susceptible individuals inflammation and IBD might be triggered (2,14,18).

In several studies, characteristic compositions of the IBD microbiome have been found, with dysbiosis being a common trait, and certain alterations being specific for CD and UC. Spanning both forms of IBD, decreased Shannon index of bacterial taxa is a common feature in comparison to healthy controls (28). Especially in CD, Shannon index shows a reduction of up to 50 %, whereas in UC it is only 30 %. The loss of diversity is due to an relative increase of anaerobic bacteria and the Firmicutes phylum (28,64).

Another interesting marker is β diversity, which compares the microbial ecosystem of one individual to the rest of a group. A popular method to demonstrate similarities of microbial compositions in different individuals is principal coordinate analysis (PCoA). The data of each person's microbiome is set into context with the study group and visualized by appointing a specific location in a diagram. Through this data analysis clustering of different microbiome subtypes is performed, whereas IBD significantly shows separate clusters compared to healthy controls. Also, CD and UC cluster in distinct groups, marking specific microbial compositions for healthy, CD and UC individuals (65).

In addition, some studies found specific bacterial strains to be enriched or deprived in IBD subtypes versus healthy controls. For example, *Fusobacterium* and *Escherichia* were found to be increased in CD, whereas almost absent in UC and healthy controls. In the same study *Collinsella* was increased in UC, but absent in CD (66). However, these differentiations in the gut microbiome are often unreliable, as microbial compositions underlie many factors, resulting in different findings and prohibiting exact microbial predictions in IBD (48,65).

Not only the bacterial composition is disturbed, but fungi, viruses and archaea are moving into focus of research with intriguing results. Fungal dysbiosis seems to be a common feature in IBD, including an increased Basidiomycota to Ascomycota ratio. In addition, some fungal

species, for example *S. cerevisiae*, are deprived, while others, mainly *C. albicans* thrive in IBD. These findings suggest, that long neglected fungi maintain significant roles in IBD pathophysiology, but further research is required to fully understand their potential in triggering and sustaining IBD (67).

Recent findings suggest a contributing role of archaea in IBD pathophysiology as well. The methane-producing *Methanosphaera stadtmanae* is a pro-inflammatory microbe and was shown to have a more than doubled prevalence in stool samples from IBD patients compared to controls (47 % versus 20%). Intriguingly, only IBD patients react with an IgG-specific response to *M. stadtmanae*, whereas healthy controls seem to have no inflammatory reaction. This suggests an important role for archaea in IBD however, further studies are needed to understand their impact on pathogenesis (68).

Further highlighting the importance of microbiota in IBD pathophysiology, is the effectiveness of fecal microbiota transplantation in UC patients. Studies have shown that transmitting stool of healthy donors to UC patients, induces remission in a subset of patients. This indicates that the microbiome might be causatively involved in UC pathogenesis (69).

1.3.4 Microbiome in IBS

Increasing evidence suggests that the microbiome is involved in IBS pathogenesis and dysbiosis is acknowledged to contribute to symptoms. Certain features of the microbiome, like depletion of some Clostridiales and enrichment of *Ruminococcus torques*, and raised Firmicutes to Bacteroidetes ratios, have been found (34,70). In animal models, IBS symptoms, including visceral hypersensitivity, altered stool transit time and increased permeability, can be transferred from IBS patients to germ-free animals through fecal transplantation, asserting the important role of the microbiome (34).

Also, IBS microbiomes tend to have a higher abundance of gas-producing bacteria, like *Dorea*, which could result in higher gas development in the intestines. This gas overproduction is associated with IBS and could be responsible for bloating, pain and flatulence (34).

Especially the microbiome of PI-IBS, which develops after gastrointestinal infection, is intriguing to examine. Resembling symptoms of IBS-D, the microbial composition of PI-IBS also matches closely with IBS-D. PI-IBS harbors high levels of Bacteroidetes phylum, whereas many *Clostridia* are decreased significantly. Also, activated bacterial genes and pathways point to higher intestinal permeability due to impaired barrier function of the epithelium (71).

Closely linked to the microbiota is food intolerance in IBS (72). By degrading fiber, bacteria produce small metabolites like short chain fatty acids (SCFAs), which are thought to have beneficial effects on the mucosa. However, in IBS fermentable oligo-, di- and monosaccharides and polyols, which are present in fiber-rich foods are not well tolerated. This may be due to gas formation occuring during digestion of fibers, which is then perceived as distension pain in hypersensitive individuals. It might also be a result of bacterial dysbiosis leading to over- or underproduction of bacterial metabolites like SCFAs, thereby causing IBS symptoms. SCAFs are known to stimulate the immune system and therefor contribute to a healthy state. However, when SCAF levels reach a certain point, they might overstimulate T cells and therefor cause low-grade inflammation (34,70).

Another way bacteria could be involved in IBS symptom development, is through serotonin (5-HT) regulation. 5-HT is a mediator controlling gut motility and is produced by enteroendocrine cells, which are stimulated by bacteria. Especially involved in this stimulation are Clostridiales, of which some strains tend to be increased in IBS, which could accelerate gastrointestinal transit time, possibly leading to diarrhea (34).

Not only bacteria are involved in IBS pathophysiology, fungi might also contribute to the disease, especially to visceral hypersensitivity. Recently, it has been shown that IBS patients harbor fungal dysbiosis compared to healthy controls, with decreased fungal diversity (Shannon index) in IBS. In addition, the two main strains of the human mycobiome, *S. cerevisiae* and *C. albicans*, seem to be even more dominant in IBS patients compared to healthy individuals. Differences in the mycobiome suggest a relevant impact of fungi on IBS, however further mechanistical studies are needed (73).

Also of interest are archaea, which have not been intensively studied in the context of IBS yet. Mainly *Methanobrevibacter* has been examined in IBS studies, showing decreased abundance in IBS patients compared to controls. In addition, the presence of methanogens correlates inversely with loose stool frequency, suggesting that this subgroup of archaea might contribute to firm stool consistency and slower colonic transit (71,74).

Fecal microbiota transplantation has also been tested for treating IBS, with inconsistent but some positive results, showing that shifts to a more diverse microbiome can improve symptoms. This points towards an important role of the microbiota in IBS pathophysiology, however microbial alterations might not alone explain disease development (75,76).

1.3.5 Microbial Biofilms

A biofilm is a group of microorganisms adhering to each other and sticking to a surface, thereby becoming more resistant to outer stressors. Built up by microorganisms, biofilms can exist as single-species films, but also form communities encompassing different kingdoms, e.g. bacteria and fungi. Growth of biofilms is an ancient and frequent, but incompletely understood form of microbial life. Through complex interaction of the microbes, the cells of a community are able to proliferate when conditions are too harsh for single cells. By forming a biofilm, microorganisms are able to adapt quickly to a changing environment and become more flexible than single cell organisms alone could be (77,78).
As an important survival strategy, biofilms are ubiquitous and occur in a variety of ecosystems, including desserts, oceans, hot springs and the human body. Single-species biofilms are less common and tend to occur mostly in medical conditions, such as *Pseudomonas aeroginosa* on medical implants and infections (77,78).

1.3.5.1 Biofilm Formation

Biofilms form in four stages, beginning with stage one when planktonic (free, floating) cells first loosely attach to a surface. In stage two this attachment become adhesive, a monolayer is formed and extracellular matrix is produced. Monolayers consist of cells only growing beside each other, not on top or beneath. In stage three, the formation of multilayers and microcolonies occurs. In stage four, maturation and growth of the biofilm takes place. A mature biofilm consists of multiple microcolonies, which are separated by water channels transporting nutrients and responsible for waste disposal. Usually biofilms take up a mushroom-like shape, but they can also remain flat or adapt to new forms. At last, in stage five biofilms detach from a surface and reverse to planktonic cell state or shed single cells which then attach to another surface to conclude the cycle (78,79).
Triggers for biofilm formation are incompletely understood, but hostile environments are likely to induce development. A combination of environmental factors and genetic disposition of the planktonic cells is most likely to attribute for biofilm organization. Outer stressor include exposure to ultraviolet light, acids, toxic metals, dehydration and drugs (78). Of special interest in this context are antibiotics, which naturally are stressors to bacteria. One study showed that a sub-inhibitory dosage of aminoglycosides was able to induce biofilm formation in *Escherichia coli* and P. *aeruginosa*. The so-called aminoglycoside

response regulator gene in *E. coli* and *P. aeruginosa* was responsible for this reaction and closely linked to surface adhesion(80).

Multiple mechanisms have been introduced how microbes benefit from biofilm communities and therefor are likely to form them. First, the extracellular matrix offers barrier protection from dehydration and antimicrobial agents. Second, biofilms tend to have dormant or starved microbes in the outer zones, protecting vital microbes of the inner center from intruders. This especially applies to antibiotics, of which many require vital cell activity to be effective. Third, there could be dormant subgroups of highly resistant microbes, which could partially protect the biofilm from stressors (78).

It has also been proposed, that biofilm formation takes place when nutrients are available and detachment occurs when nutrient scarcity arises within the biofilm (77).

1.3.5.2 Biofilms in Human Disease

As stated above, biofilms occur universally and have been recognized as pathogenic in many human diseases. Biofilm associated illnesses range from infected kidney stones, infectious endocarditis, cystic fibrosis and dental plaque to the infection of medical implants and catheters. Treatment of biofilm infections poses challenges to clinicians, since the microorganisms harbor a secure matrix and being less metabolically active, protecting them from antibiotics (77–79).

In gastroenterological context, biofilms have recently gained some acknowledgment as well. A striking new finding revealed the presence of microscopic colonic biofilms in CRC, possibly involved in cancer induction. In the study by *Dejea C. et al*, 89% of right-sided tumors harbored bacterial biofilms, which stretched to far distant healthy mucosa, whereas left-sided tumors had surprisingly few biofilms (12 %). These biofilms were present on benign polyps and CRC, evidence that they develop before cancer arises. Signs of reduced cell adhesion and pro-inflammatory state were associated with biofilms, with reduced E-cadherin in epithelial cells, elevated IL-6 levels and increased cell proliferation. These findings suggest that bacterial biofilms might activate cellular pathways towards faster proliferation and tumor development (81). The results were confirmed by two other studies, supporting that bacterial biofilms are a characteristic feature in right-sided colonic tumors (50,82).

Recently, specific bacterial species have been identified to dominate these biofilms, the majority of them rather associated with dominating the oral cavity than the intestines. *Bacteroides fragilis, Fusobacterium nucleatum, Streptococcus stomatis* and *Parvimonas*

micra were especially enriched in biofilm samples (82). *F. nucleatum* has been recognized as a cancer-related microbe before, being present in inflamed mucosa and on colon tumors.(83,84) Another study of CRC in familial adenomatous polyposis also found biofilms on tumors, confirming *B. fragilis* and identifying *E. coli* as another dominant strain (50).
The concept of pathologic biofilms in the gastrointestinal tract has also been suggested for gastritis, with *Helicobacter pylori* biofilms in patients with failed eradication therapies.(85)
In a study by *Swidsinski et al*, microscopic biofilms were found in up to 95 % of IBD patients, 95% of self-limiting colitis patients, 65 % of IBS patients and 35 % of healthy controls. Overall bacteria tended to be adherent to the mucosal wall and consist of different species. *B. fragilis*, which has also been associated with tumor biofilms, was found to be strongly represented in IBD biofilms, however far less abundant in IBS biofilms. The study found biofilms to be suppressed, but not completely eliminated by antibiotics and mesalazine. This poses evidence that bacterial biofilms might play an important role in IBD and IBS pathophysiology. Spatial organization of microbial communities in the human intestine has been a neglected research topic (86).

1.3.5.3 Bacterial-Mucosal Interaction

Molecular interactions of bacteria and epithelial cells at the mucosal surface is of particular interest. It is likely that many factors, including bacteria, immune cells, epithelial cells, their mediators and environmental toxins need to be disturbed to cause chronic inflammation and eventually tumorigenesis. These interactions at the mucosal barrier are also likely to play an important role in biofilm formation (85,87).
Cancer pathogenesis is often induced by chronic inflammation, which is also the case for CRC. Although the exact cellular mechanisms are incompletely understood, immune cells and their mediators are thought to escalate DNA damage in epithelial cells and inflammation induces higher cell turnover, increasing the accumulation of DNA mutations. Chronic inflammation is also known to alter microbiota composition and may also affect the properties and abilities of commensal bacteria. In a mouse model for example, the commensal *E. coli* was found to act more aggreassivly in an inflammatory setting of the colon, using polyketide synthase to induce invasive carcinomas when inflammation was already present (88).
Another study found that cytolethal distending toxin of gram-negative bacteria may increase genomic instability and therefor induce mutations in epithelial colon cells. These examples

are just a few pathways of how bacterial behavior is influenced by its surroundings and how microorganisms might be involved in cancer pathogenesis (89).

Of central importance to microbial and host interaction is the mucosal barrier with mucus secreted by goblet cells, which forms a physical barrier between the colon lumen and the epithelium. Mucus consists mainly of mucin, glycoproteins and other proteins, including IgA and antimicrobial peptides and is central to intestinal homeostasis (87). A thin inner mucus layer lies directly on the producing epithelial cells, whereas the outer layer is more loose and colonized by bacteria. The inhabiting bacteria co-regulate the mucus production and show different transcription pathways compared to the same species in the middle of the colon lumen (90).

Multiple common gut bacteria are able to target and degrade mucus glycoproteins as an energy source. Especially when dietary fiber is scarce, bacteria rely more on mucin, thereby reducing the mucus layer (91). Recently, *Ruminococcus gnavus* was found to adhere to the mucus layer and to degrade sialic acid sugars to form substrates only possible to be used by *R. gnavus* itself (92). In addition, some bacteria are able to make direct, close contact to the epithelial cells. As an example, *B. fragilis* is able to occupy intestinal crypts this way (87).

Some pathogenic and commensal bacteria, like *Clostridium perfrigens*, *R. gnavus* and *Bacteroides caccae*, feature enzymes to disrupt the mucosal barrier, thereby gaining entrance to the intestinal epithelium and sparking inflammation by provoking immune cells (87).

Possessing flagella seems to be a mechanism to adhere to mucus as well, which is how *Clostridium difficile* is suspected to form biofilms in the intestines. Details on how and why bacteria succeed to form biofilms in the intestine are still lacking, however two functions have been recognized as crucial: the ability to adhere to the mucosa and to move freely through mucus (93). The expression of adhesion factors has been recognized as central to biofilm formation. The factors used for adhering, including type 1 pili, curli amyloid fibrils and cellulose, also stimulate immune cells and thereby trigger inflammation (94). In addition, when bacteria accomplish invading the inner mucus layer, they seem to disrupt epithelial cells and tight junctions in their integrity. This is thought to result in increased epithelial permeability, thereby impeding epithelial barrier function. This barrier disruption could hence allow pathogens and toxins to enter the epithelium and to cause an inflammatory process there (87,95). This mechanism is suspected to be a key factor in IBD pathogenesis. Individuals with impaired mucus production (due to genetic variations) plus a low-fiber diet might be more susceptible to commensal bacteria degrading their mucus layer, which could

in turn allow toxins and pathogenic bacteria to reach the epithelium and cause inflammation there. This hypothesis aligns with findings in UC patients, who lack mucus on inflamed districts, whereas this has not been shown for CD (87,96).

A mechanism of how bacteria are triggered to start biofilm formation might be BS concentration. BA are produced in the liver and secreted into the small intestine to support digestion and uptake of fats. When passing through the terminal ileum, almost all BA are reabsorbed, only a small percentage is eliminated with feces. A meta-analysis found that 58 % of IBS-D patients have BA malabsorption, which could lead to diarrhea (97). Interestingly, it has also been reported that the regulation of BA synthesis and their salts is disturbed in some IBS patients, especially in IBS-D. The same study found higher amounts of BA in stool to be associated with diarrhea and IBS-D. In addition, a substance to induce BA synthesis, 7α-hydroxy-4-cholesten-3-one, was found to be upregulated in 38% of IBS-D patients, which did not occur in IBS-C and controls (98).

Linking BA malabsorption with biofilm formation could explain some of IBS pathophysiology. To understand how bacteria are affected by BS, *Pumbwe et al.* conducted an experiment with *B. fragilis*, which is a common bacterium in gastrointestinal infections and has been linked to biofilms. By incubating *B. fragilis* with BS, the bacterium started overproducing membrane vesicles and appendages similar to fimbriae, used for adhesion. In fact, BS-treated bacteria were more resistant to antimicrobial agents, adhesion within the bacterial colony, but also with intestinal epithelial cells and biofilm formation increased. Although these findings were made *in vitro*, they could also apply to IBS patients, who have higher BA levels in their intestines, disrupted BA homeostasis and biofilm formation could be part of IBS pathophysiology (99).

1.3.5.4 Polymicrobial Interaction and Biofilms

As mentioned earlier, microbiome studies often focus on bacteria, neglecting the impact of fungi and archaea. Especially in the context of gastrointestinal biofilms, an understudied research area, data on polymicrobial biofilms is lacking.

First of all, when studying polymicrobial biofilms, understanding interactions between different microorganisms is essential. Fungi and bacteria both occur in the intestines, but also in many other body sites. When antimicrobial therapy is performed, e.g. with antibiotics, microbial imbalances might occur which leads to *Candida* infection. The commensal and highly abundant fungus only acts as a pathogen, when bacterial composition is depleted and *C. albicans* is therefor able to spread, or when the immune system is impaired (100).

However, fungal-bacterial interactions go much further: It has been shown that fungi are able to control behavior and survival of bacteria, and vice versa, bacteria impact fungal physiology and growth. In addition, bacterial and fungal virulence is mediated through polymicrobial interactions. Secreted molecules are used for polymicrobial communication, by signaling population density and suggesting decrease of proliferation. Also, some bacteria harbor antifungal toxins, and some fungi produce antibacterial substances – the discovery of penicillin is one of the most significant examples (100,101).

An interesting example of bacterial-fungal interaction was demonstrated in a recent study of colitis. In a mouse model, colitis was triggered with dextran sodium sulfate, and microbial composition determined the severity of inflammation. The adding of probiotic *Saccharomyces boulardii* attenuated inflammation, whereas *C. albicans* escalated it. Intriguingly, Enterobacteriaceae were necessary for fungi to be able to modulate colitis severity. This is an example of how bacteria and fungi interact complexly, which is likely to occur in biofilms as well (102).

Investigating polymicrobial biofilms, additional bacterial-fungal interactions have been revealed. Antagonistic interactions have been found, when bacteria form a biofilm on a fungal hyphae or yeast cell, suppressing the fungus by secreting antifungal toxins. Bacteria may also alter pH level, thereby impairing fungal hyphae formation (101).

In contrast, cooperating fungal-bacterial interactions also exist, e.g. in polymicrobial biofilms, when different microorganisms offer protection against immune mediators and antimicrobial substances (100). Evidence points at higher stress resistance, acceleration of biofilm growth and metabolic collaboration, when genetic variation is high, increasing the fitness of the microbial community. These symbiotic interactions might occur in gastrointestinal biofilms and could contribute to resistance and disease pathophysiology (103).

Polymicrobial biofilms are insufficiently studied, more data is available on single-species fungal biofilms, which pose challenges in clinical context. Most importantly, fungal biofilms occur on medical implants, catheters and devices, whereas fungi in general cause infections when the immune system is weakened. Many fungal species have been described to cause infection and create biofilms in human disease, with *C. albicans* being the leading microbe (104). The physiology of fungal biofilms resembles the one of bacteria, with only slight differences. To build a biofilm, the most important asset is the ability to adhere to a surface. As stated above, fungal biofilms have mostly been found on artificial surfaces, however they

also exist on tissues, including teeth, vessels and mucosa. Molecules facilitating adhesion of fungi include glycoproteins, encoded by the agglutinin-like sequence gene family, cell wall proteins and surface proteins (104–106).

Many genes involved in biofilm development are encoding for transcription factors and kinases, regulating genetic expression and protein synthesis. Of certain interest in this context is the production of extracellular matrix, which affects biofilm vitality and resistance to environmental stressors (106,107).

Specific for fungal biofilms are the different cell morphologies used by fungi. When initiating a biofilm, yeast cells adhere to a surface and start proliferating. During stage two, initiation of the biofilm, fungal cells form filamentous shapes, building hyphae and pseudohyphae – an ability exclusively present in fungi. Upon these observations, the hypothesis that both appearances, yeast cells and hyphae, must be present to accomplish fungal biofilm maturation, has been formed. In addition, a big part of genes relevant for biofilm development are related to hyphae creation, emphasizing the importance of this cell type (106,107).

Another interesting feature of biofilms is the mechanism used for communication within the biofilm, known as quorum sensing, which has been described in both bacterial and fungal contexts. In order to coordinate the behavior of a biofilm community, quorum-sensing molecules are secreted by bacterial or fungal cells which than act upon target genes in neighboring cells. Through this mechanism, gene expression for biofilm adhesion, morphology of cells, cell proliferation and shedding of biofilm is regulated and adapted to the environment (103,108). A well-studied quorum-sensing molecule in fungal biofilms is farnesol, which inhibits filamentation and therefor impairs biofilm growth. Farnesol is an abundant molecule in biofilms and likely to be involved in dispersion of fungal cells (106).

Few data are available on archaeal biofilms or their interactions with the intestinal mucosa and other residing microbes. However, it has been shown that the predominant archaea, *M. smithii*, is able to make close contact to the intestinal epithelium. Also, *M. smithii* modulates bacterial efficiency in digestion of polysaccharides, thereby influencing calorie extraction of dietary intake. In order to persist in the colon, *M. smithii* produces adherence structures and certain surface glykans resembling the ones of the mucosa and is able to sustain itself from bacterial fermentation products. These findings suggest that *M. smithii* is able to adhere to the mucosa, a key ability in order to form biofilms (109).

Supporting evidence comes from an in-vitro study, where biofilm development of archaea was examined. The strains *M. smithii* and *M. stadtmanae*, both residing in the gut microbiome, were found to form multilayer biofilms on mica and uncoated plastic dishes, with *M. smithii* reaching up to 40 µm height. This clearly shows that archaea are able to form biofilms, possibly also in the intestines (110).

Biofilms consisting of bacteria and archaea have also been discovered, however they are mostly studied outside of the human body. Methanogenic archaea and sulfate reducing bacteria occur as symbiotic partners in biofilms, in which archaea oxidate methane and bacteria use the released electrons in order to reduce nitrate and sulfate. This mechanism demonstrates the ability of archaea and bacteria to cooperate as polymicrobial biofilms, suggesting that the same process could occur in the intestines as well (111).

1.4 Hypothesis

In our study cohort of 385 patients undergoing colonoscopy, we discovered for the first time macroscopically visible biofilms adhering firmly to the intestinal mucosal wall of a subgroup of patients. When categorizing by disease phenotype, we found that 47 % of IBS and 27 % of UC patients, whereas only 11% of gastroenterological asymptomatic controls harbored biofilms (112).

Biofilms are an extremely common phenomenon in nature, providing microorganisms with protection from outside stressors and making them more resistant in hostile environments. Bacteria, fungi and archaea are all able to form single-species or polymicrobial biofilms, residing as symbionts in the latter (78,105,111).

In human disease context, microscopic bacterial and fungal biofilms have been associated with dental plaque, medical implants, endocarditis and many more disorders. Specifically in CRC, microscopic bacterial biofilms have been causally linked to pathogenesis and are suspected of inducing tumor growth (113).

Many microorganisms have been associated with a variety of diseases, especially disorders of the gastrointestinal tract, including IBD and IBS. By adhering to the intestinal epithelium, thereby impairing the mucosal barrier and sparking an inflammatory process, some bacteria have been associated with IBD pathogenesis (87).

We hypothesize that the intestinal biofilms found in our cohort consist of not only bacteria, but also fungi and archaea. Moreover, we hypothesize the microbial biofilms to be pathogenic and contribute to disease pathophysiology of IBD and IBS. Furthermore, we suspect biofilm composition to be resembled in matching stool samples. The aim of this

study is to determine microbial composition (archea and fungi) of stool and biofilm samples from IBD, IBS and control patients to gain an understanding of their role in disease pathophysiology.

2 Materials and Methods

The goal of this study was to examine the microbial composition of stool samples (ST) and biofilm flushes (BFF) in IBD, IBS and control patients.

In order to realize this, we established a DNA extraction method and performed polymerase chain reaction (PCR) to detect fungi, archaea and bacteria in STs and BFFs. Amplicons were subjected to sequencing to analyze microbial composition of STs and BFFs. Statistical analysis was performed using R, in order to detect differences between patient groups and between biofilm positive and negative patients.

2.1 Patient Cohort and Samples

The original study encompassed 385 patients, who underwent colonoscopy at the Allgemeines Krankenhaus, AKH, Medical University of Vienna. When analyzing for patient groups, we found a high prevalence of biofilms in IBS (47%) and UC (27%), whereas only 11% of controls and 6 % of CD patients harbored biofilms (112).

To investigate these groups further and understand microbial composition in these individuals, we examined a selected subgroup of 72 samples from 49 patients in this study. Focusing on fungal and archaeal data, we analyzed 43 STs and 29 BFFs.

We analyzed 31 UC, 23 IBS, 15 control, two IBD-U samples and one stomach BFF from a patient with Barrestt's esophagus. Out of all samples, 20 pairs were established, consisting of ten UC, seven IBS and three control pairs. In Table 1 and Figure 1, the patient cohort is depicted.

ST samples were collected in the outpatient clinic. BFFs were collected from patients during colonoscopy in the gastroenterological outpatient clinic for endoscopy by flushing with Jetstream using physiological sodium chloride. Both ST and BFFs were stored at – 80 ° C.

The ethic commission of Medical University of Vienna gave ethical approval for collecting and analyzing STs and BFFs (ethical commission number 1617/2014). Informed consent of patients was given.

For fungi and archaea all 72 samples were extracted, sequenced and analyzed. For bacteria, only BFFs were analyzed.

Table 1. Sample distribution.

	BFF	**ST**	**Total (pairs)**
UC	14	17	31 (10)

IBS	8	15	23 (7)
Control	4	11	15 (3)
IBD-U	2	0	2 (0)
Barrett's esophagus	1	0	1 (0)
total	29	43	72 (20)

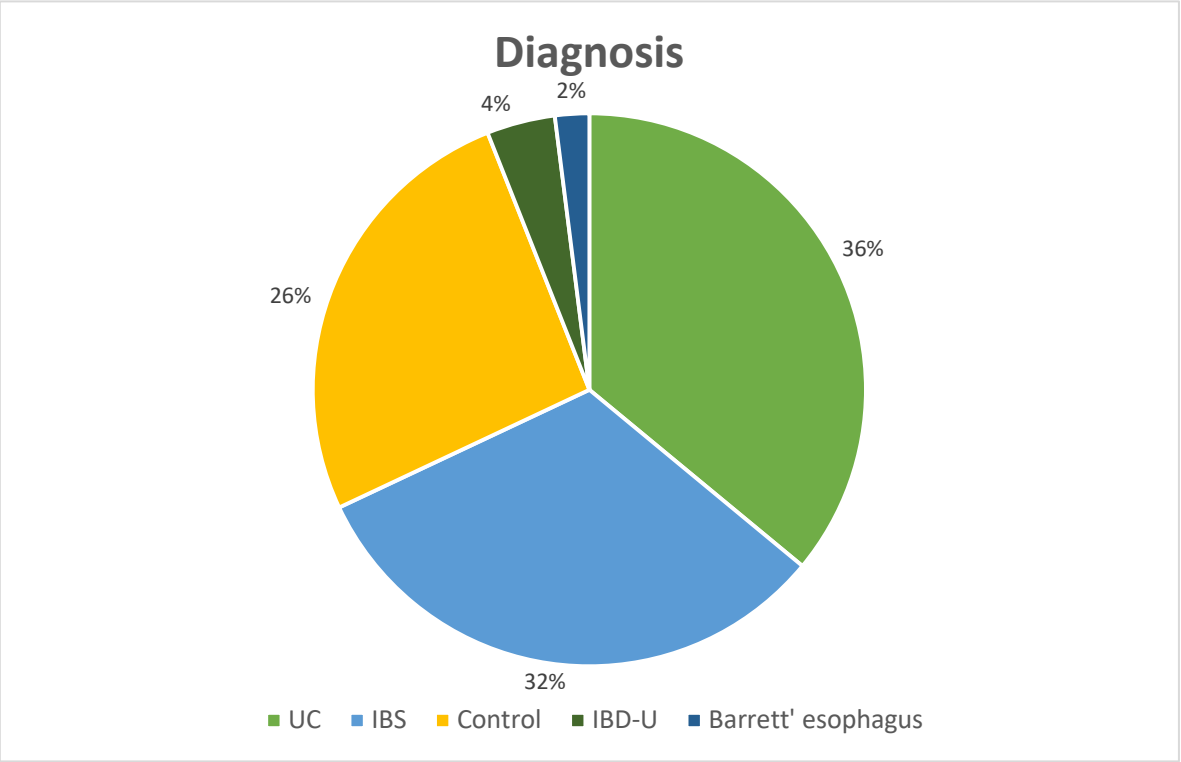

Figure 1: Patient cohort.

2.2 Establishing a High Quality DNA Library of STs and BFFs

2.2.1 DNA Extraction

Since fungi have cell walls in addition to cell membranes, breaking the cells to extract fungal DNA is more challenging than gaining access to bacterial DNA. Therefore, protocols aimed at bacterial DNA are insufficient in fungi. In order to receive optimal results, we tested two separate extraction protocols and evaluated DNA output.

First, an adapted extraction protocol for RNA and DNA by *Griffiths et al.* was used (114). Therefore, phenol-chloroform-isoamyl alcohol and bead beating at 5700 rpm for one minute in total were used for lysing cell walls. The exact protocol can be found in Supplement 1.

Next, we tested the International Human Microbiome Standards DNA extraction protocol Q (IHMS SOP 06 V1: Standard Operati ng Protocol for fecal Samples DNA Extraction) using the Qiagen QIAamp DNA Stool Kit. Key to cell wall lysis was heavy bead beating at 6500 rpm for one minute at a time, repeated 16 times with 5 minutes of rest in between (115). We extended the incubation time overnight with isopropanol, dissolved the nucleic acid pellet in AE-Buffer of the Qiagen DNA Stool Mini Kit and centrifuged spin columns for longer times than in the protocol. The adapted protocol can be found in Supplement 2.
After DNA extraction, DNA concentration of samples was measured with Nanodrop 1000 (Thermo Fisher, Waltham, Massachusetts).

2.3 1st PCR

In the next step, extracted samples were processed by PCR to screen for fungal and archaeal DNA in STs and BFFs. PCR for bacteria was only conducted in STs. Fungal and bacterial PCR were done at the Christian Doppler Laboratory for Molecular Carcinoma Chemoprevention at Medical University of Vienna, whereas archaeal PCR was conducted at the Center for Microbiome Research at the Medical University of Graz. For the first PCR, primers with Illumina overhang adapters were used (Table 2).

Table 2. Illumina overhang adapters.

Adapter	Sequence
Forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
Reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

2.3.1 Fungi

Since microbiome studies focusing on fungi are scarce, optimized methods for detection of fungal DNA are lacking. To attain the best results possible, we tested two sets of primers and varied PCR cycle amount. Primers can be found in Table 3.

Table 3. Fungal PCR Primers

Primer name	Primer sequence
ITS3_KYO2	GATGAAGAACGYAGYRAA
ITS4_KYO3	CTBTTVCCKCTTCACTCG
ITS1-30F	GTCCCTGCCCTTTGTACACA

ITS1-217R	TTTCGCTGCGTTCTTCATCG

PCR primers to screen for fungal DNA should have wide taxonomic coverage to detect all fungal organisms, regardless of which fungal species are present in samples. To achieve this coverage, PCR primers targeting a genetic region coding for ribosomal clusters were used. For fungi we used the Internal Transcribed Spacer (ITS) region. We used different ITS primers and evaluated their efficiency.

The first set of primers were ITS4_KYO3 and ITS3_KYO2, which were kindly provided by Athanasios Makristathis, DI, PhD, Associate Professor of Clinical Microbiology, (Department of Clinical Microbiology, Medical University Vienna).

Different primer concentrations were tested, with 0.2 µM and 0.4 µM primer per sample. We also varied the number of cycles, including 30, 35 and 40 cycles of PCR. In addition, different DNA concentrations were selected, with experiments of 15 ng, 50 ng, 100 ng and 200 ng template DNA per sample.

Next, we evaluated a second set of fungal primers, which had recently been reported to achieve 79,9 ± 7,1 % of taxonomic coverage (116). ITS1-30F and ITS1-217R were used in 0.2 µM concentration per sample. Template DNA concentration was again evaluated in different concentration, including 15 ng, 50 ng, 100 ng and 200 ng. Cycle number was also tested with 30, 35 and 40 cycles per PCR.

All PCR products were evaluated by 2 % agaroses-gel electrophoresis (Biozym LE Agarose, Biozym Scientific, Germany), using 1 µL of GelRed/ 100 µL (Biotium, USA) and ChemiDoc MP Imaging System (Bio-Rad, UK) to detect PCR product bands. We used 12.5 µl of 2X GoTaq Green Master Mix (Promega, USA) and depending on sample volume, added DNase-free water to reach 25 µl volume per sample. In Table 4 final fungal PCR methods are summarized.

Table 4. Fungal PCR methods.

Established PCR methods	
Reagents	**Concentration**
DNA template	100 ng
Primer ITS1-30F	0.2 µM
Primer ITS1-217R	0.2 µM
2X GoTaq Green Master Mix	12.5 µl

Time	Temperature
3 min	95° C
	40 Cycles of
30 sec	95° C
30 sec	55° C
2 min	72° C
10 min	72° C
∞	12° C

2.3.2 Archaea

In order to detect archaeal DNA, we performed nested PCR targeting the archaeal 16S rRNA gene. The first PCR was conducted using the primer 344aF and 1041R, which are archaea-specific and therefore increase archaeal DNA abundance compared to interfering bacterial DNA. In the second PCR, the primers Illu 519F and Illu 806R were used, which amplify universal 16S rRNA genes, also present in bacteria. Due to first increasing archaeal 16S amplicons, archaeal 16S is highly abundant and therefore it is unlikely that 16S of other origin is amplified. In Table 5 primer sequences are depicted (117).

Table 5. Archaeal PCR primers.

Primer name	Primer sequence
344aF	ACGGGGYGCAGCAGGCGCGA
1041R	GGCCATGCACCWCCTCTC
Illu 519F	CAGCMGCCGCGGTAA
Illu 806R	GGACTACVSGGGTATCTAAT

For both PCRs, TAKARA Ex Taq buffer with MgCl2 (Takara Bio Inc., Tokyo, Japan), BSA (Roche Lifescience, Basel, Switzerland), dNTP mix, TAKARA Ex Taq Polymerase and water (Lichrosolv®; Merck, Darmstadt, Germany) were used. In Table 6 and Table 7, PCR procedures and reagents are listed.

Table 6. First archaeal PCR methods.

Reagents	Concentration
DNA template	5 µl
Primer 344aF	10 µM

Primer 1041R	10 µM
TAKARA ExTaq Buffer with $MgCl_2$	10 X
BSA	20 mg/ml
dNTP mix	2.5 mM
ExTaq Polymerase	5 U/µl
Time	**Temperature**
5 min	95° C
	25 Cycles of
30 sec	94° C
45 sec	56° C
1 min	72° C
10 min	72° C

Table 7. Second archaeal PCR methods.

Reagents	**Concentration**
DNA template	5 µl
Primer 519F	10 µM
Primer 806R	10 µM
TAKARA ExTaq Buffer with $MgCl_2$	10 X
dNTP mix	2.5 mM
Ex Taq Polymerase	5 U/ µl
Time	**Temperature**
5 min	95° C
	40 Cycles of
40 sec	95° C
2 min	63° C
1 min	72° C
10 min	72°C

PCR products were evaluated by 2 % agaroses-gel electrophoresis (Biozym LE Agarose, Biozym Scientific, Germany), using 1 µL of GelRed/ 100 µL (Biotium, USA) and ChemiDoc MP Imaging System (Bio-Rad, UK) to detect PCR product bands.

2.3.3 Bacteria

To detect bacterial presence in BFFs, we performed bacterial 16S rRNA PCR, targeting the V3-V4 region with primers 341F and 785R. Primer sequences are depicted in Table 8 (118).

Table 8. Bacterial PCR primers.

Primer name	Primer sequence
341F	CCTACGGGNGGCWGCAG
785R	GACTACHVGGGTATCTAATCC

For PCR we used 12.5 µl of 2X GoTaq Green Master Mix (Promega, USA), 0.2 µM of both primers, 50 ng of template DNA and depending on sample volume, we added PCR-grade water to reach 25 µl volume per sample. 25 cycles of PCR amplification were performed. PCR products were evaluated by 2 % agaroses-gel electrophoresis (Biozym LE Agarose, Biozym Scientific, Germany), using 1 µL of GelRed/ 100 µL (Biotium, USA) and ChemiDoc MP Imaging System (Bio-Rad, UK) to detect PCR product bands. In Table 9, final fungal PCR methods are summarized.

Table 9. Bacterial PCR methods.

Reagents	Concentration
DNA template	50 ng
Primer 314F	0.2 µM
Primer 785R	0.2 µM
2X GoTaq Green Master Mix	12.5 µl
Time	**Temperature**
3 min	95° C
	25 Cycles of
30 sec	95° C
30 sec	55° C
1 min	72° C
7 min	72° C
∞	4° C

2.4 Preparation for Sequencing

After concluding PCR runs, all PCR products were prepared for sequencing. This includes a second PCR stage with Index Illumina Primers, which attach to the amplified region of the

Illumina overhang adapters. After each PCR, a clean-up step was performed. Before MiSeq Sample Loading, library quantification and normalization were conducted. Ilumina 16S Metagenomic Sequencing Library Preparation Guide was used as protocol for the following steps (119).

2.4.1 1st PCR Clean-up

To remove free primers, primer dimers and DNA fragments before proceeding to the second PCR stage, we performed a PCR clean-up step. On a 96-well plate, PCR products were incubated with 20 µl AMPure XP beads (VWR International, Radnor, USA) per sample. AMPure XP beads bind DNA fragments 100 bp and larger, leaving primers, primer dimers and DNA fragments. In a next step the 96-well plate was placed on a magnet, causing AMPure XP beads with the binding DNA amplicons to be pulled to the well ground. Next the supernatant with the waste floating in it, was removed. DNA amplicons with AMPure XP beads were washed twice with 70% ethanol and left to air-dry for ten minutes. Removing the plate from the magnet, amplicons were dissolved in 52.5 µl of 10 mM Tris (pH 8.5) per sample, eluting them form AMPure XP beads. After incubation the plate was again placed on the magnet, and dissolved DNA amplicons now floating in the supernatant were transferred to a new PCR 96-well plate (120).

2.4.2 2nd PCR

For the second PCR step or index PCR, 5 µl of cleaned DNA amplicons of each sample were distributed in a 96-well plate, which was then placed on a TruSeq Index Plate Fixture. Twelve Nextera XT Index Primers 1 were set up on the horizontal side of the plate fixture and eight Nextera XT Index Primers 2 on the vertical side. Adding 5 µl of a primer to its corresponding row, an individual primer content was established for each sample, marking them specifically. Also, 10 µl PCR grade water and 25 µl 2x KAPAHiFi HotStart ReadyMix were added to each sample. Next, Index PCR was performed (119). In Table 10, Index PCR methods are listed.

Table 10. Index PCR.

Reagents	Volume
Cleaned DNA amplicons	5 µl
Nextera XT Index Primer 1 (N7xx)	5 µl

Nextera XT Index Primer 2 (S5xx)	5 µl
2x KAPAHiFi HotStart ReadyMix	25 µl
PCR grade water	10 µl
Time	**Temperature**
3 min	95° C
	8 Cycles of
30 sec	95° C
30 sec	55° C
30 sec	72° C
5 min	72° C
∞	4° C

2.4.3 2nd PCR Clean-up

After the Index PCR, a second washing step was performed. Except for using 56 µl of AMPure XP beads to remove DNA fragments, primers and primer dimers, and dissolving Index PCR amplicons in 27.5 µl of 10 mM Tris pH 8.5 afterwards, the protocol did not differ from the first PCR clean-up (119).

2.5 Sequencing

Library Quantification and Normalization of DNA amplicons were conducted in a standardized matter, as determined by the 16S Metagenomic Sequencing Library Preparation Protocol. Next, the library was denaturized as designated by the protocol and samples were loaded to MiSeq (Illumina, San Diego, USA). (119) This process and MiSeq sequencing were performed in cooperation with the laboratory of Clinical Microbiology (Athanasios Makristathis, DI, PhD, Department of Clinical Microbiology, Medical University Vienna). Arachael sequences were retrieved from the Core Facility Molecular Biology at ZMF, Medical University of Graz.

2.6 Analysis

Analysis of sequencing reads was conducted using DADA2 and SINA (121). Reads were analyzed as amplicon sequence variants (ASVs), which are orginal DNA sequences retrieved from sequencing. To assign taxonomy to ASVs, the Unite Database was used. For verification of fungal taxonomy, the most abundant reads were double-checked by BLAST of NCBI. Sample similarity was analyzed with modified Rhea scripts. Visualization of generalized Unifrac distances was performed with non-metric Multi-Dimensional Scaling

(NMDS) at ASV level. To assess cluster significance, we used permutational multivariate analysis of variance. To compare relative abundance of taxa, Kruskal-Wallis Rank Sum Test was applied and to adjust p-values for multiple comparisons, Bonferroni correction was used. As additional readout we performed LEfSe (linear discriminant analysis effect size) analysis, using the galaxy server of *Huttenhower lab* (http://huttenhower.org/galaxy), using standard setting (one-against-all strategy) (122).

3 Results

3.1 Fungal Methods evaluation

Since microbiome studies focusing on fungal composition are scarce, we aimed to identify the best methods for extraction and detection of fungal DNA. In order to evaluate efficiency of existing methods and to gain best results, we investigated different experimental settings and protocols in a subset of our ST and BFF samples.

As described in 2.2.1, we compared two different DNA extraction protocols for fungi. The extraction protocol for RNA and DNA by *Griffiths et al* (114), using phenol-chloroform-isoamyl alcohol and little bead beating (5700 rpm for one minute in total), revealed inferior to the IHMS DNA extraction protocol Q (115), which used intense bead beating (6500 rpm for one minute at a time, repeated 16 times). DNA concentrations were similar with both extraction methods, however, when performing ITS PCR afterwards, only 6/12 versus 10/12 samples were positive for fungi, using the *Griffiths et* al and IHMS protocols, respectively. This suggests, that in order to break fungal cell walls, mechanical lysis is superior to chemical lysis. Therefore, we continued using the IHMS protocol Q for extraction of all 72 samples.

When evaluating fungal PCR methods, we also tested different approaches. Comparing two sets of fungal ITS primers ITS4_KYO3 and ITS3_KYO2 versus ITS1-30F and ITS1-217R, we obtained more positive results (20/36 versus 33/36) with the latter. Especially in BFFs, which tended to have lower DNA concentrations in the first place, ITS1-30F and ITS1-217R were more successful in detecting fungi. Because of that, we settled for ITS1-30F and ITS1-217R for all samples prepared for sequencing. However, amplification bands with ITS1-30F and ITS1-217R were not always regularly shaped, but sometimes migrated to different levels in gel electrophoresis (Figure 2 and 3).

Experimenting with different PCR cycle amounts, we found 35 cycles to be sufficient to detect distinct amplification bands in STs, however not in BFFs. To achieve standardized samples, we hence settled for 40 cycles in both STs and BFFs. In Figure 2 and Figure 3, results of PCRs using the different primers are shown.

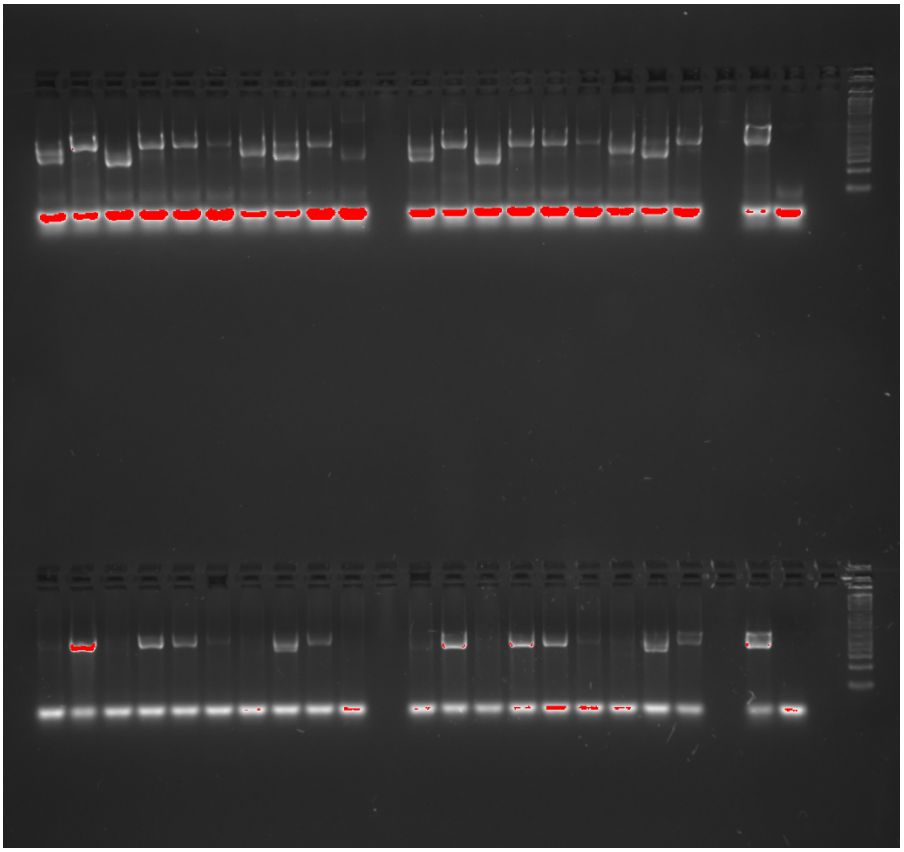

Figure 2. Fungal PCR of STs using 35 cycles.

ITS1-30F and ITS1-217R *(top lanes)*, ITS4_KYO3 and ITS3_KYO2 *(bottom lanes)*.

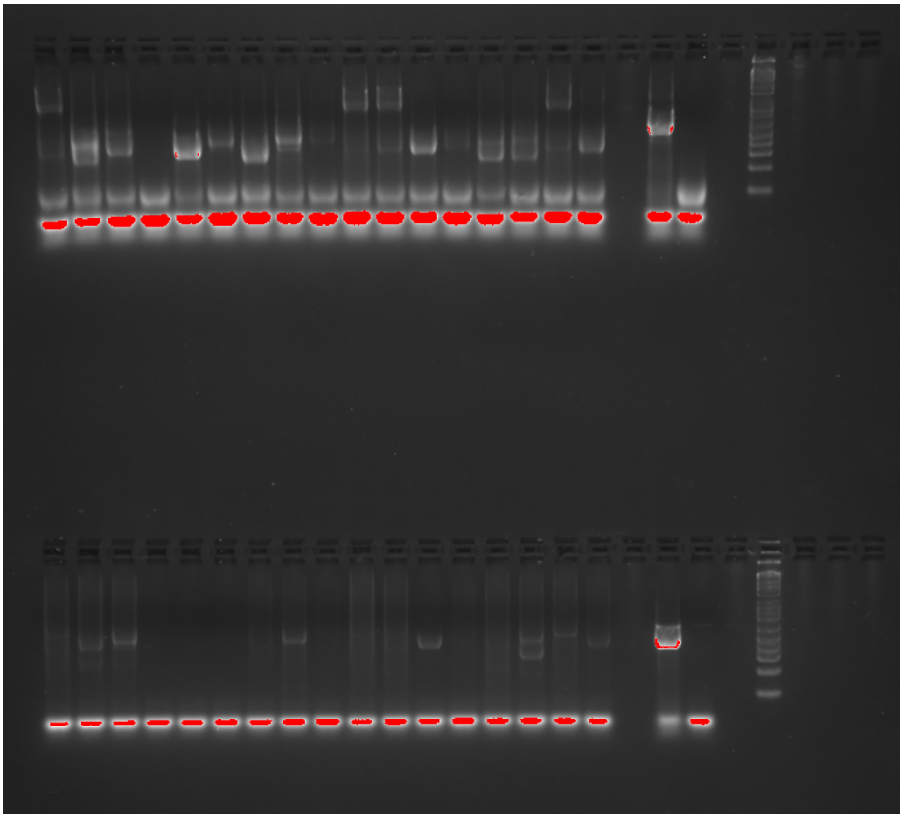

Figure 3. Fungal PCR of BFFs using 40 cycles.

ITS1-30F and ITS1-217R *(top lanes)*, ITS4_KYO3 and ITS3_KYO2 *(bottom lanes)*.

3.2 Microbial Composition of STs and BFFs

To identify a distinct microbiome of feces and biofilms in IBD and IBS, we analyzed the microbial composition of biofilms and stool samples of all patient cohorts. We aimed to detect compositional differences between patients groups, between samples from BFF-positive versus BFF-negative patients, as well as define microbial alterations between BFF and ST samples. To get rid of unspecific (non-fungal/ -archaeal/ -bacterial) sequences, an alignment and phylogenetic tree were calculated for each kingdom. Obvious outliers were removed manually.

3.2.1 Fungal composition of STs and BFFs

First, we aimed to detect fungi in all STs and BFFs using PCR. Out of all 29 BFFs, 25 were found positive for fungal DNA, whereas out of all 43 STs, 37 showed positive results. Nevertheless, all samples were next put into sequencing.

When analyzing the abundance of fungal orders in STs and BFFs after sequencing, 89 ASVs were found by Unite Database, of which a considerable amount was either uncultured or unknown. Therefore, we corrected fungal taxonomy results with BLASTN and found eight

of the thirty most abundant reads to be either eukaryotes or plant cells, probably due to nutritional intake and unspecific amplification (see Table 11). The corresponding DNA reads are shown in Supplement 4.

Table 11. Most abundant fungal ASVs, correction with BLASTN.

Sample ID	Kingdom	Phylum	Class	Order	Family	Genus	Species	Match
ASV1	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Candida	albicans	99
ASV3	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Candida	albicans	100
ASV29	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium	tenuissimum	99
ASV12	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Dipodascaceae	Galactomyces	candidum / geotrichum	98/ 99
ASV7	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	roqueforti / carneum	99 / 99
ASV9	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Dipodascaceae	Galactomyces	candidum / geotrichum	98 /99
ASV2	Eukaryota	Heterokonta	Blastocystae	Blastcystida	Blastocystidae	Blastocystis	hominis	97
ASV48	Eukaryota	Amoebozoa	Tubulinea	Tubulinida	Hartmannellidae	Hartmannella	verminformis	99 / 99
ASV4	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Dipodascaceae	Galactomyces	geotrichum	99
ASV17	Fungi	Uncultured fungus, clone ck-104 18S ribosmal RNA gene						75
ASV89	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Candida		99
ASV5	Eukaryota	Heterokonta	Blastocystae	Blastcystida	Blastocystidae	Blastocystis	hominis	97
ASV13	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Candida		100
ASV100	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Eurotium		99
ASV39	Fungi	Uncultured fungus, clone ck-104 18S ribosmal RNA gene						
ASV75	Eukaryota	Uncultured eukaryote clone CMH403						99
ASV80	Plantae	Angiosperms	Eudicots	Asterids	Apiales	Apiaceae	Angelica	99
ASV22	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Dipodascaceae	Galactomyces	candidum / geotrichum	98 / 100
ASV31	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Eurotium		100/ 100
ASV108	Plantae	Angiosperms	Asterids	Solanales	Solanaceae	Solanum	lycopersicum	99
ASV37	Fungi	Uncultured fungus, basidomycetes						99
ASV11	Eukaryota	Uncultered eukaryote						100
ASV20	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Dipodascaceae	Galactomyces	candidum/ geotrichum	98/100
ASV107	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Candida	albicans	99
ASV21	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Candida	dubliniensis	100
ASV6	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Candida		99
ASV77	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium	parasubtilissimum	99
ASV55	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium	tenuissimum	100
ASV194	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Aspergillus	candidus	99
ASV177	Eukaryota	Uncultured eukaryote						

The corrected composition of STs and BFFs for the most abundant fungi at order level is depicted in Figure 4 (after elimination of unspecific reads). In both STs and BFFs the most abundant fungus was *Candida albicans*.

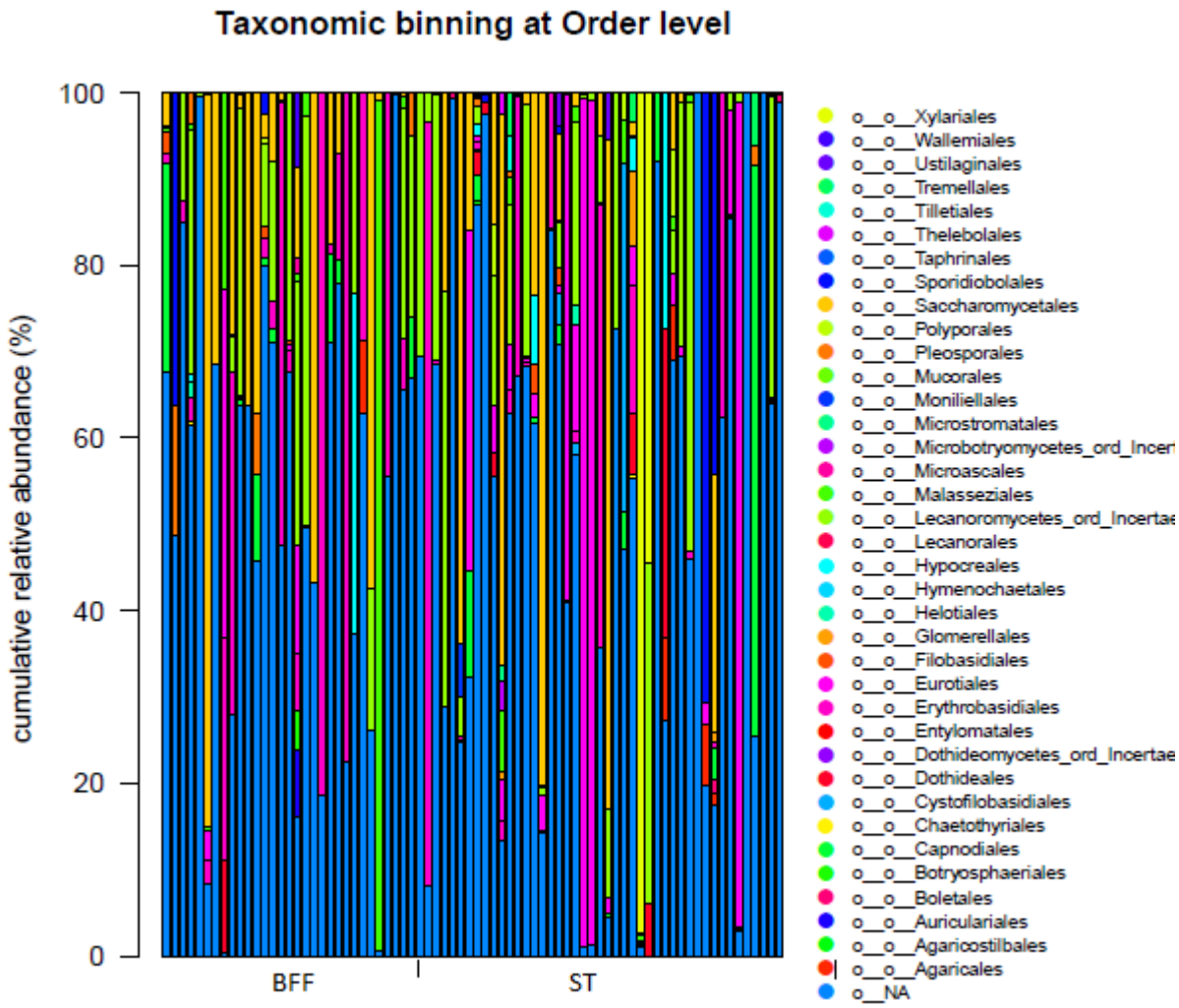

Figure 4. Most abundant fungi in BFF and ST.

Relative fungal composition of BFFs *(left)* and all STs *(right)*.

Then we compared fungal composition at ASV level in BFFs with STs of biofilm-positive patients (ST-BF-pos), using NMDS plots for depiction. BFFs and ST-BF-pos showed a big overlap in their fungal composition, illustrating that fungi present in stool are mostly present in biofilms as well (Figure 5).

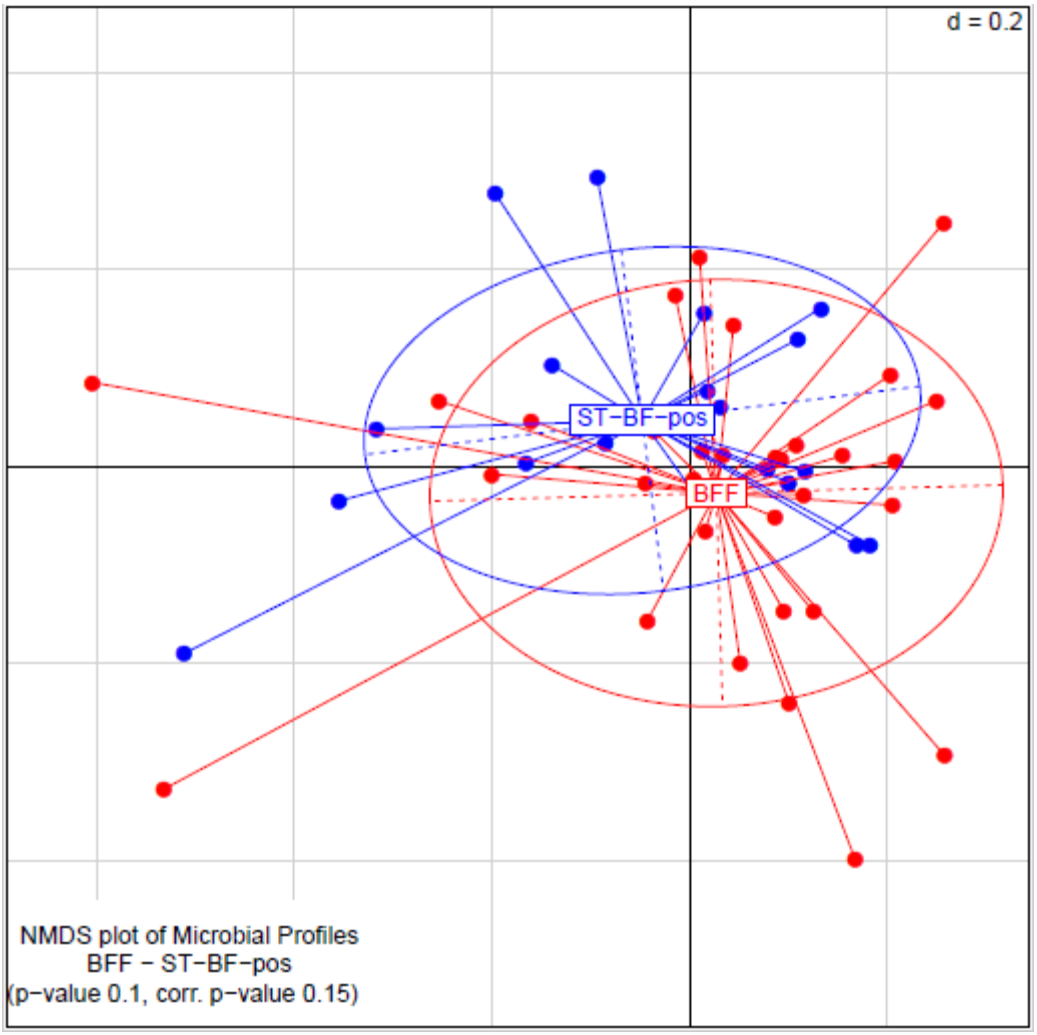

Figure 5. Fungal composition: BFF vs. ST-BF-pos.

Comparison of fungal composition in biofilms *(red)* and stool of biofilm-positive patients *(blue),* depicted using NMDS plots. Each dot represents a sample.

To determine if biofilm feature can be predicted by fungal stool composition, we performed a comparison of STs of biofilm-negative patients (ST-BF-neg) versus ST-BF-pos at ASV level, again using a NMDS plot (Figure 6). Some differences in fungal composition were visible, however not distinct nor significant.

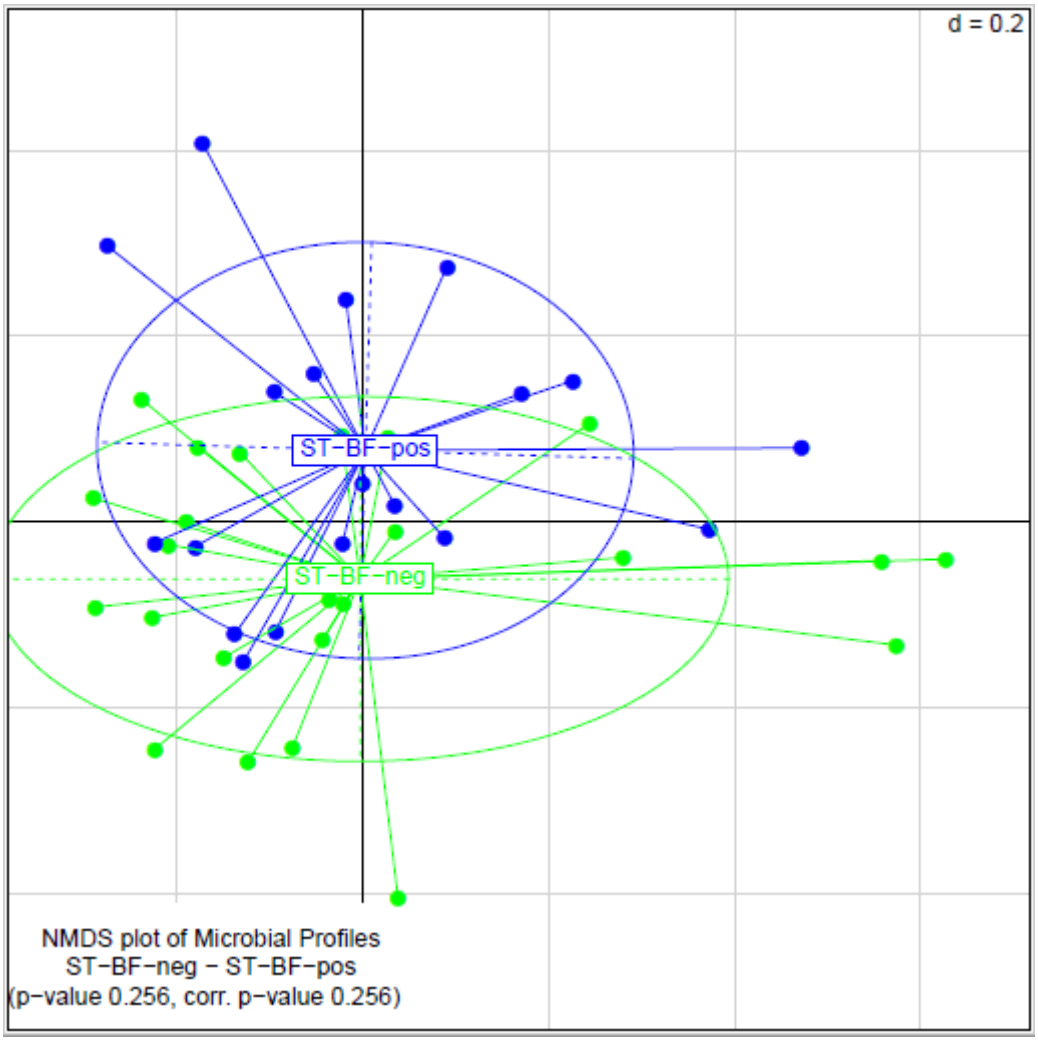

Figure 6. Fungal composition: ST-BF-neg vs. ST-BF-pos.

Comparison of fungal composition in stool of biofilm-negative *(green)* and stool of biofilm-positive patients *(blue),* depicted using NMDS plots. Each dot represents a sample.

Next, we evaluated the compositional similarities in BFFs and ST-BF-neg at ASV level and found significant differences in NMDS plots (Figure 7). This demonstrates that fungal composition in stool of individuals without biofilms lacks resemblance to the composition in biofilms.

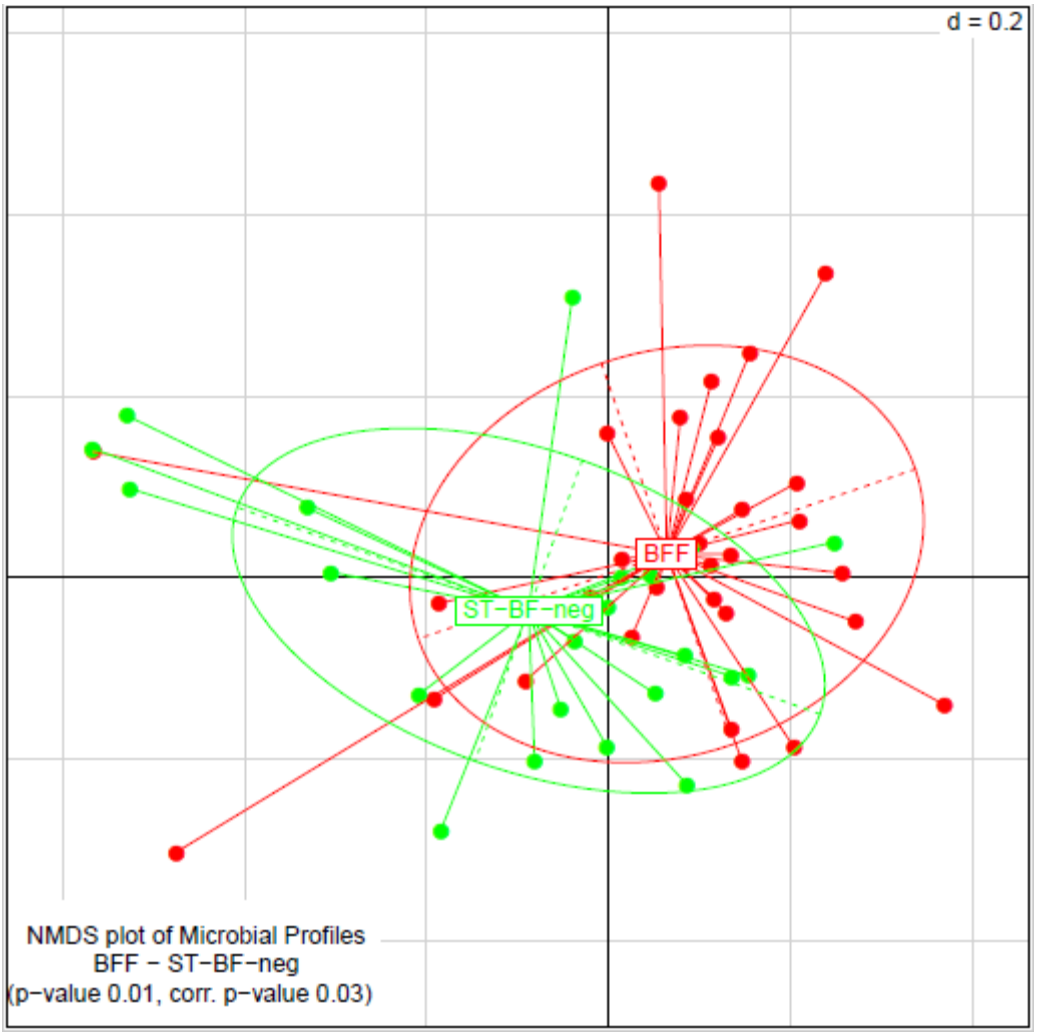

Figure 7. Fungal composition: BFF vs. ST-BF-neg.

Comparison of fungal composition in biofilms *(red)* and stool of biofilm-negative patients *(green),* depicted using NMDS plots. Each dot represents a sample.

When comparing the fungal BFF composition between disease cohorts, we did not detect any significant differences between IBS, IBD or controls (Supplement 3).

LEfSe analysis on fungal data of ST-BF-pos vs. ST-BF-neg revealed Hypocreales, Basidomycota and Agaricales to be significantly enriched in ST-BF-pos samples (Figure 8).

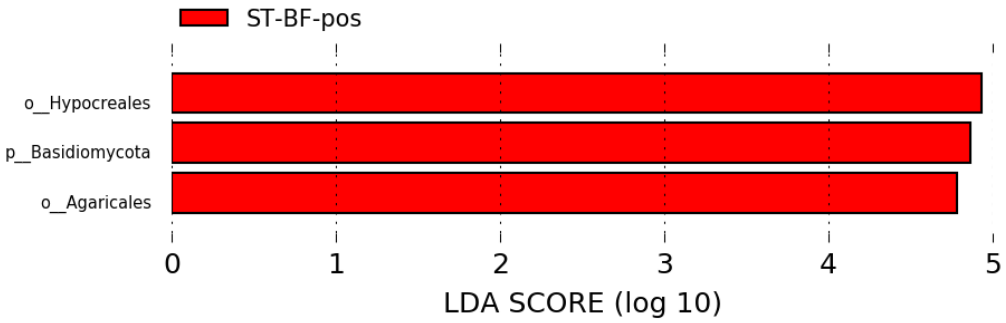

Figure 8. Fungal LEfSe analysis: ST-BF-pos vs. ST-BF-neg.

Visualization of fungal LEfSe analysis of ST-BF-pos and ST-BF-neg, ranked according to effect size.

3.2.2 Archaeal composition of STs and BFFs

First, we used PCR to detect archaea in all STs and BFFs. Out of all 29 BFFs, 25 were positive for archaeal DNA, whereas out of all 43 STs, 38 contained archaeal DNA.

When analyzing the abundance of archaeal orders in STs and BFFs after sequencing, 91 ASVs were found. However, richniss was low, because of many ASVs stemming from the same strains. The list of the overall most abundant archaea is depicted in Table 12.

After sequencing all samples, 20 BFFs and 18 STs were found positive for archaea.

Table 12. Most abundant archaeal ASVs.

Kingdom	Phylum	Class	Order	Family	Genus	Species
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	Methanobrevibacter smithii TS146C
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	Methanobrevibacter smithii TS146C
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	Methanobrevibacter smithii TS146C
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	Methanobrevibacter smithii TS146C
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	Methanobrevibacter smithii TS146C
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanosphaera	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanosphaera	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrososphaerales	Nitrososphaeraceae	uncultured thaumarchaeote	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	Methanobrevibacter oralis
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	Methanobrevibacter oralis
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	Methanobrevibacter smithii TS146C
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	uncultured rumen methanogen M6
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	Methanobrevibacter smithii TS146C
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	Methanobrevibacter smithii TS146C

The composition of BFFs and STs for the most abundant archaea at genus level is depicted in

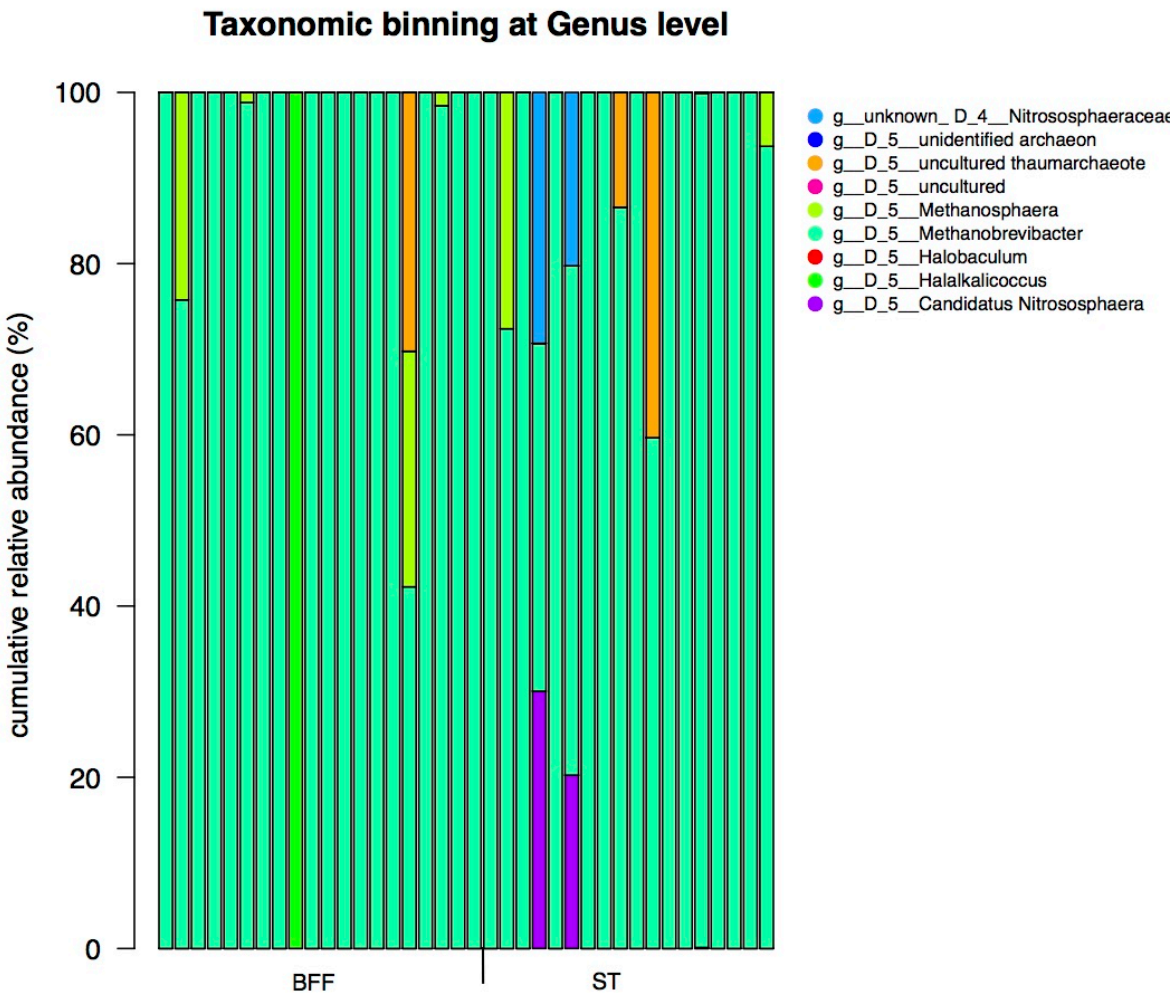

Figure 9. *Methanobrevibacter* was present in all ST samples, and all but one BFF sample of the positive samples. *Methanosphaera* was the second most common genus in BFFs, with presence in 4 out of 20 BFFs. In STs an uncultured *Thaumarchaeote*, *Candidatus Nitrosphaera* and an unknown *Nitrosphaeracaceae* genus were the second most abundant.

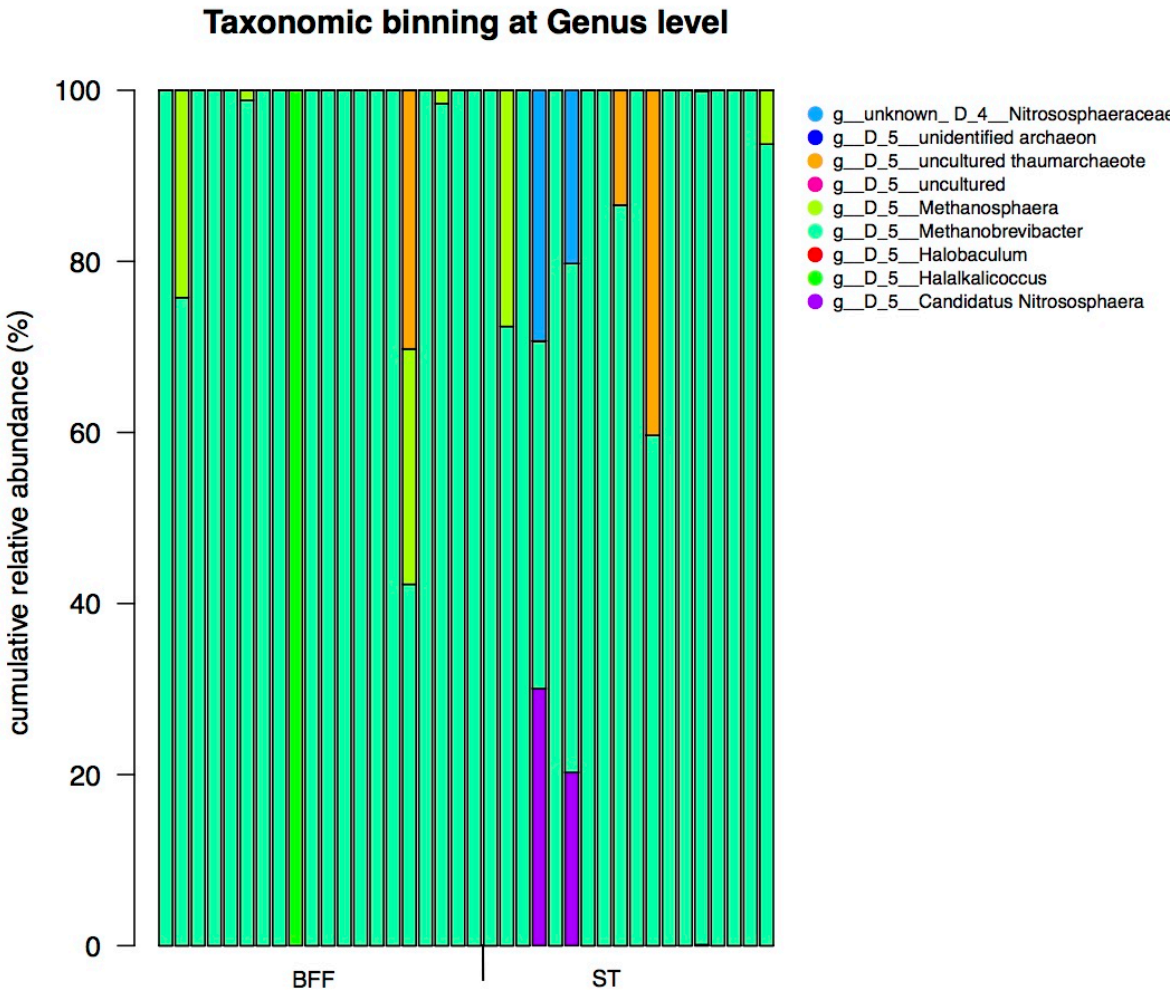

Figure 9. Most abundant archaeal genera.

Relative archaeal composition of BFFs *(left)* and STs *(right)*.

When studying archaeal composition of samples, we found BFFs and ST-BF-pos to overlap closely, showing similar archaeal profile at ASV level (Figure 10).

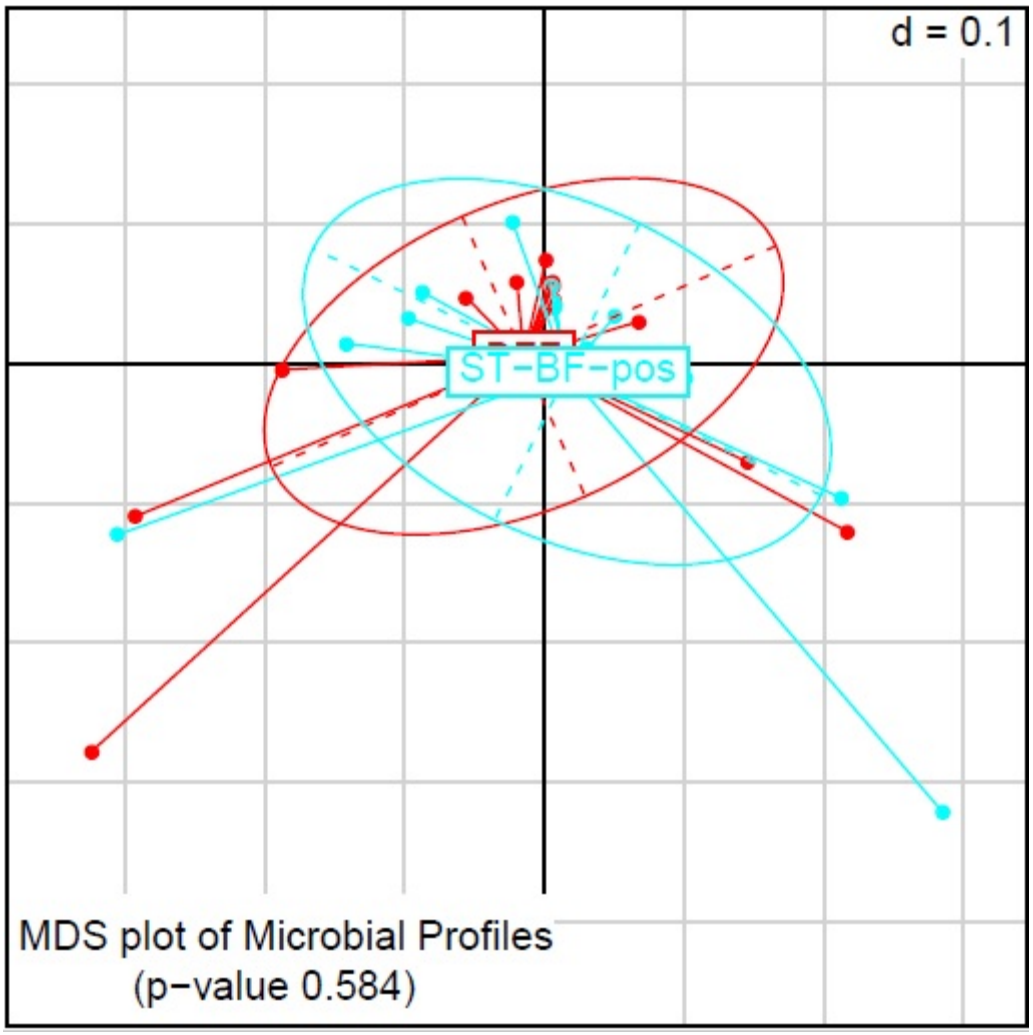

Figure 10. Archaeal composition: BFF vs. ST-BF-pos.

Comparison of archaeal composition in biofilms *(red)* and stool of biofilm-positive patients *(turquoise),* depicted using NMDS plots. Each dot represents a sample.

Next, we compared STs of patients who harbor biofilms versus patients who do not (ST-BF-neg versus ST-BF-pos) at ASV level and found significant differences in archaeal composition. This suggests that biofilm feature is linked to stool composition and could be predicted by checking archaeal stool profile (Figure 11).

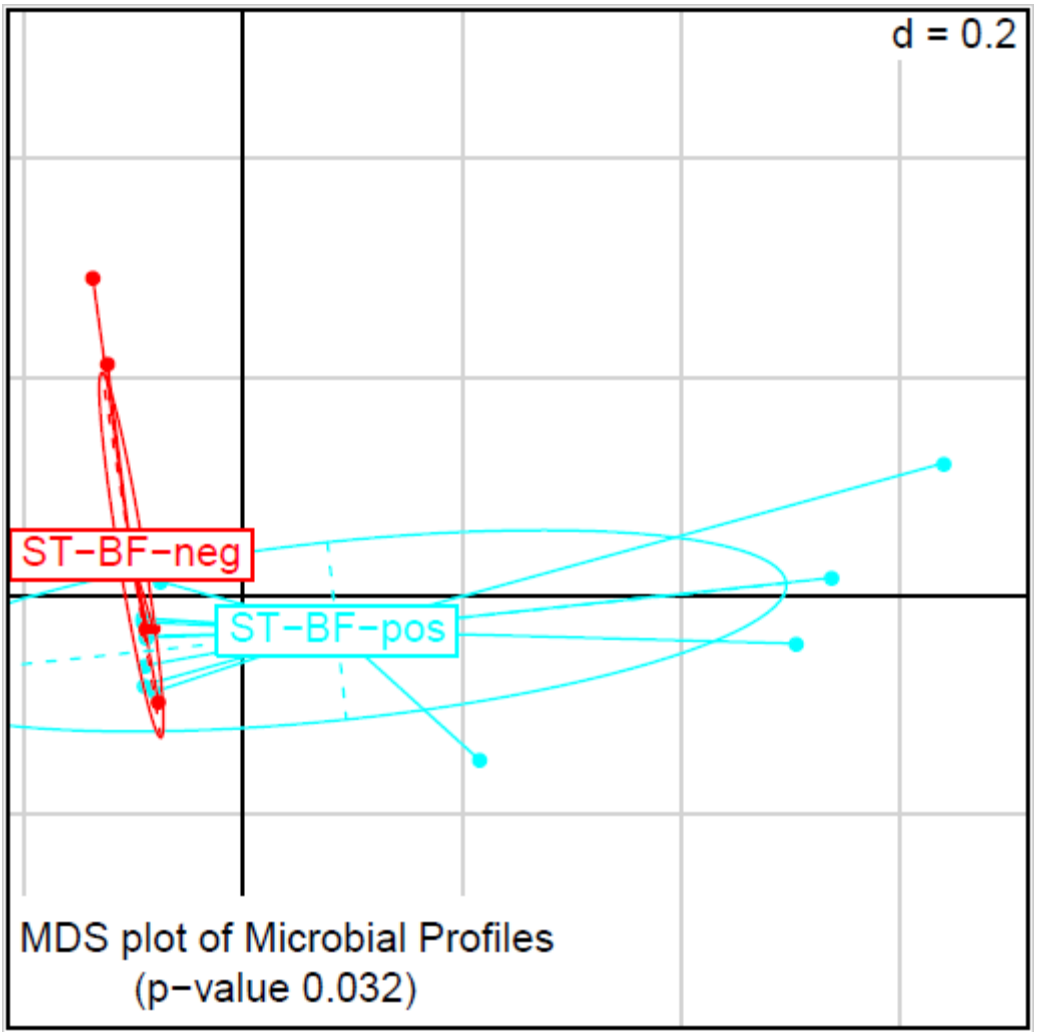

Figure 11. Archaeal composition: ST-BF-neg vs. ST-BF-pos.

Comparison of archaeal composition in stool of biofilm-negative patients *(red)* and stool of biofilm-positive patients *(turquoise),* depicted using NMDS plots. Each dot represents a sample.

In addition, we studied archaeal composition between disease cohorts in STs (sample groups were too small for BFFs). However, we did not detect any significant clustering of disease groups (Supplement 5).

LEfSe analysis of archaeal data revealed *Methanobrevibacter smithii (TS146C)* to be more abundant in ST-BF-pos than in ST-BF-neg samples (Figure 12).

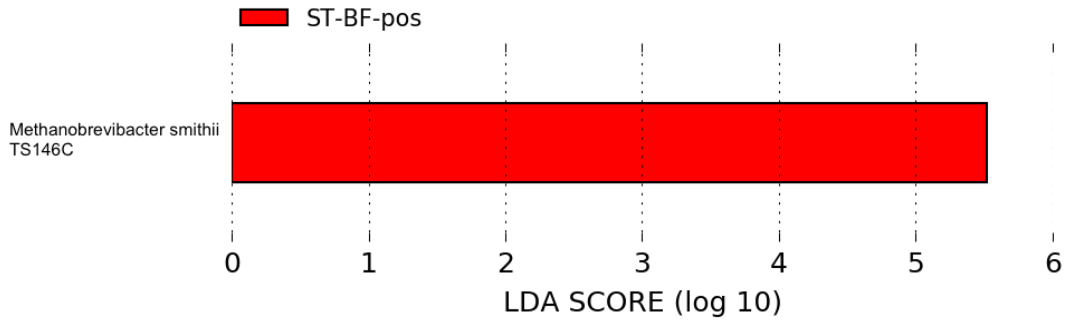

Figure 12. Archaeal LEfSe analysis: ST-BF-pos vs. ST-BF-neg.

Visualization of archaeal LEfSe analysis of ST-BF-pos and ST-BF-neg.

3.2.3 Bacterial composition of BFFs

First, when checking for bacterial DNA in all BFFs using PCR, we found 27 of all 29 samples to be positive.

When analyzing the abundance of bacterial orders in BFFs after sequencing, 973 ASVs were found. The most abundant taxa both in UC and IBS were Lachnospiraceae.

When comparing bacterial composition between diseases, we found significant clustering in IBS versus UC at ASV level (Figure 13). This shows that biofilms in IBS and UC differ from each other, a feature we could not detect when studying fungal and archaeal composition.

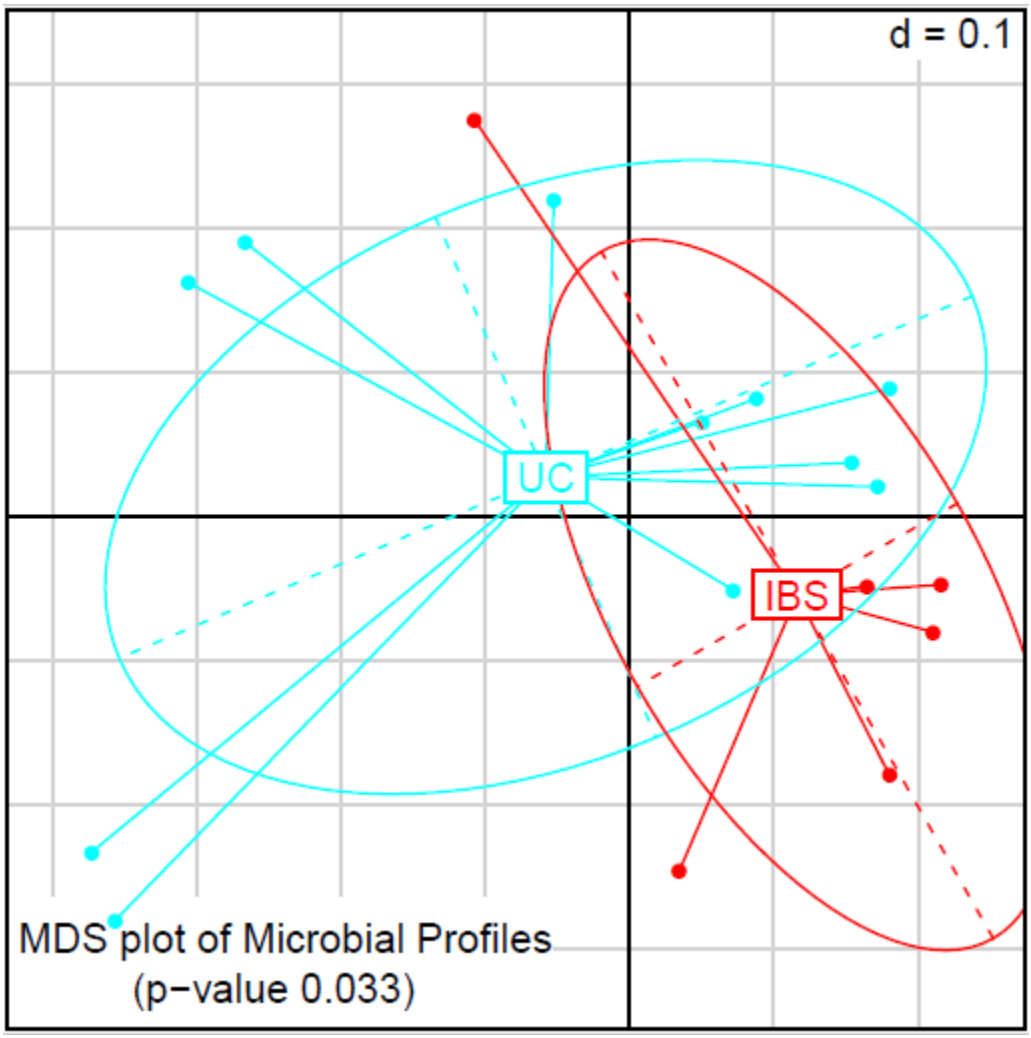

Figure 13. Bacterial composition: IBS vs. UC.

Comparison of bacterial composition in biofilms of IBS patients *(red)* and UC patients *(turquoise),* depicted using NMDS plots. Each dot represents a sample.

When looking at bacterial taxonomy of BFFs, we found significant differences of certain orders and genera between UC and IBS. For instance, *Firmicutes* were more abundant in BFFs of UC than IBS (Figure 14). In addition, we saw an increase of Erysipelotrichia (subclass), Erysipelotrichiales (order) and Erysipelothrichaceae (family) in BFFs of UC compared to IBS (Figure 15). Interestingly, Christensenellaceae (and its specific strain R7) were abundant in BFFs of UC, whereas completely depleted in IBS (Figure 16). However, *Lachnoclostridium* was increased in BFFs of IBS compared to UC (Figure 17). Even though p-values for the above mentioned strains showed significance, corrected p-values were not significant, probably due to small sample size.

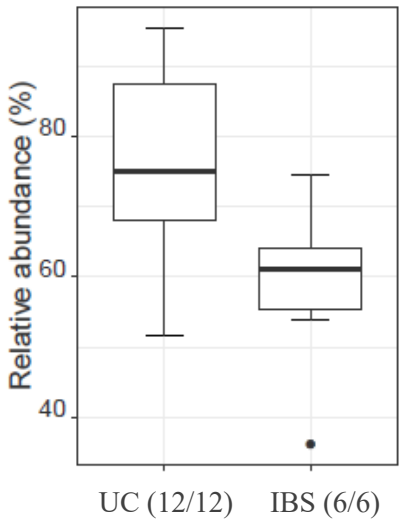

Figure 14. Firmicutes in BFFs.

p-value 0.0246, adjusted p-value 0.2141

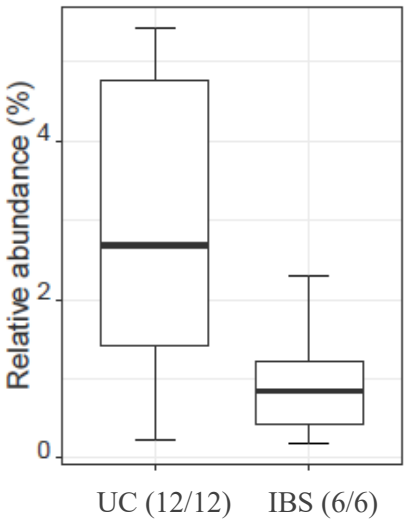

Figure 15. Erysipelothrichaceae in BFFs.

p-value 0.0192, adjusted p-value 0.2141

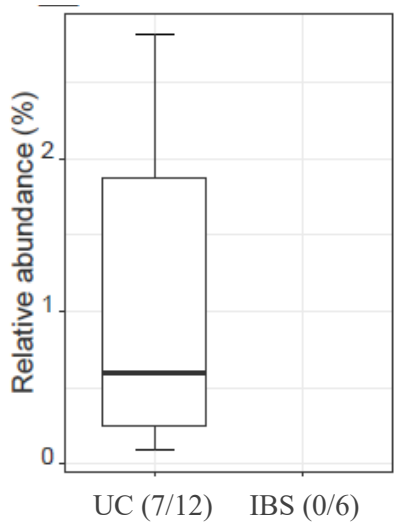

Figure 16. Christensenellaceae in BFFs.

p-value 0.0377, adjusted p-value 0.9615

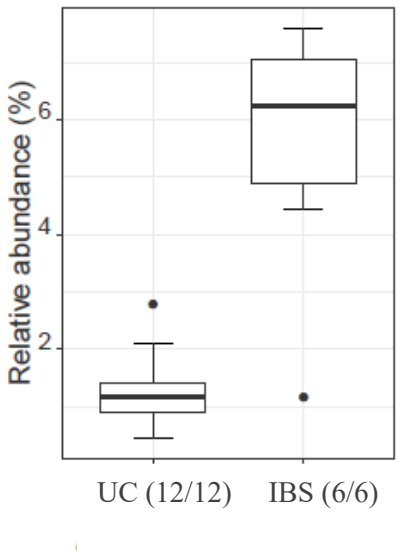

Figure 17. Lachnoclostridium in BFFs.

p-value 0.005, adjusted p-value 0.2141

LEfSe analysis of bacterial data revealed that Firmicutes and Eggerthellaceae are significantly enriched in UC compared to IBS biofilms.

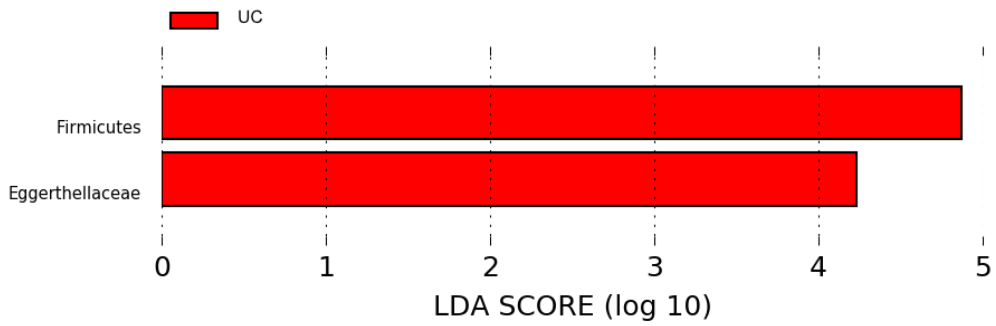

Figure 18. Bacterial LEfSe analysis: IBS vs. UC.

Visualization LEfSe analysis of IBS and UC biofilms, ranked according to effect size.

3.2.4 Stomach BFF

In a patient with Barrett's esophagus, we found a biofilm located in the stomach – something not detected in any other patient so far. When analyzing bacterial content of the BFF, we found *Streptococcus* to be the dominating genus (68% relative abundance) (Figure 19, A). *Gemella* (7%), *Granulicutella* (6%) and *Veillonella* (4%) were as well quite abundant. Testing for fungi, Lecanoromycetes (52%) and Sordariomycetes (40%) were the most abundant classes present in biofilm (Figure 19, B). Archaeal composition was not evaluated.

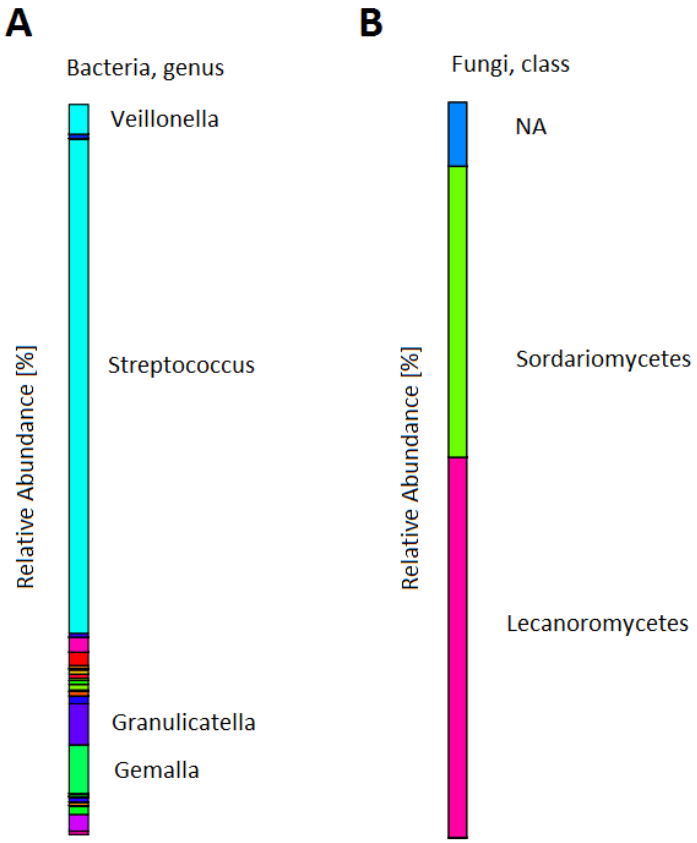

Figure 19. Taxonomic binning of stomach biofilm.

A. Bacterial genera in stomach biofilm. **B.** Fungal classes in stomach biofilm.

4 Discussion

Many studies have contributed to gaining knowledge of IBD and IBS pathogenesis. However, etiologies of both diseases remain incompletely understood. Microbiome studies are promising, showing that the gut microbiota of IBS and IBD patients differ significantly from healthy individuals. These dysbiotic conditions are thought to contribute to triggering disease. In this study, we analyzed the microbial profiles of macroscopically visible biofilms in IBD and IBS in order to detect differences in composition which might lead towards novel hypotheses on disease pathomechanism. We hypothesize biofilms to harbor a distinct polymicrobial community and to resemble their matching stool samples. Furthermore, we suspect biofilms to be drivers of IBS and possibly UC pathogenesis. BFFs and STs were analyzed for fungal, archaeal and bacterial content and comparisons were drawn between patient cohorts.

The main findings of the study comprise, that biofilms are polymicrobial microenvironments consisting of fungi, archaea and bacteria, and biofilm composition resembles the microbiome of matching stool samples in fungi and archaea. However, fecal mircrobiomes of patients with and without biofilms only show significant differences in archaeal composition, not in fungi. Significant biofilm compositions for disease phenotypes could only be detected when studying bacteria, not for fungi and archaea. Finally yet importantly, a gastric biofilm was studied and revealed high abundance of *Streptococcus* and Lecanoromycetes.

In this study, we evaluated the role of fungi in both IBD and IBS. Biofilms and stool composition of patients with biofilms (BFFs vs. ST-BF-pos) revealed high similarity, which shows that the fungi present in stool are also present in biofilms. This suggests a correlative relation between fungal stool composition and biofilm feature. This hypothesis is supported by significant differences in fungal composition of BFFs and ST-BF-neg, which demonstrates that stool of patients without biofilms does not resemble the fungal structure in BFFs. However, when comparing fungal stool composition (ST-BF-pos versus ST-BF-neg), no significant differences were detected, undermining a correlative relation between fungi in stool and biofilm feature. When further examining differences between stool samples of patients with and patients without biofilms (ST-BF-pos versus ST-BF-neg), Hypocreales, Basidomycota and Agaricales were found to be enriched in biofilm-positive patients, explaining the observed profiles of stool samples.

Fungal dysbiosis is a common, but long-underestimated feature, when studying the microbiome of IBD and IBS patients. Specific alterations in fungal composition have been described for both diseases before in previous studies. Examples include shifted Basidiomycota to Ascomycota ratio and thriving of *C. albicans* in IBD (67), increased levels of *S. cerevisiae* and *C. albicans* in IBS (73) and increased abundance of anti-*S. cerevisiae* antibodies in CD (32). Interestingly, an increase in Basidomycota in stool of biofilm-positive patients was found in this study, aligning with the previous findings of increased Basidomycota to Ascomycota ratio in IBD patients. This could be an indication that Basidomycota are not only more abundant in the IBD mycobiome, but might also contribute to biofilm formation in these individuals. However, in this study no differences between disease cohorts were found.

Polymicrobial biofilms of the gastrointestinal tract have not been studied before, therefore information on the fungal role in biofilms is lacking in the literature. To better understand the impact of fungi in intestinal biofilms in IBD and IBS, further studies are needed, since results of this study are not completely satisfying.

The inconsistencies of fungal results in our study could be due to methodologic imperfections, too small sample size or correlative relations might not exist at all between stool and biofilm feature.

Therefore, to better examine fungal impact on the gut and to achieve conclusive results in future studies, larger sample size with the possibility to form a reliable amount of matched pairs and better-developed methods are required. The lack in fungi-focused methodology is due to protocols and methods being developed and optimized for bacteria detection, rather than focusing on all sorts of microbes.

This shortage of fungi-specialized technology reduces the output and quality of fungal results, which is reflected in our experiments evaluating fungal DNA extraction and amplification via PCR. This is mirrored in our comparison of the two primer pairs for fungal amplification. We chose to use ITS1-30F and ITS1-217R rather than ITS4_KYO3 and ITS3_KYO2, since the first achieved more positive amplification bands. However, unspecific amplification bands were abundant and after sequencing many non-fungal, unspecific reads turned out to be amplified. This was emphasized when checking the most abundant fungal reads detected by Unite Database with BLASTN, where eight out of thirty reads turned out to be either of plant or blastocystis origin. These results highlight how unspecific amplification via PCR with ITS1-30F and ITS1-217R was and suggests that the chosen primers also amplified other eukaryotic DNA fragments. In future studies, using

ITS4_KYO3 and ITS3_KYO2 would therefore be recommended, since they obtained less positive, but likely more specific results.

Another contributing factor to the difficulties of detecting fungi appropriately is that the total amount of fungi is largely outnumbered by the amount of bacteria. Since fungi tend to be a lot bigger in size than bacteria, their presence in the gut might be underestimated by measuring DNA amount and not accounting for cell size.

In addition, high amplification cycle number for fungi is another obstacle in receiving specific results. For fungal DNA amplification we had to use 40 cycles, whereas 25 cycles were sufficient for bacterial DNA, with the latter reducing the amplification of unspecific reads. Although filtering fungal reads twice, results were still not specific enough as reflected by the large amount of eukaryotic sequences.

Furthermore, the fungal composition of samples may be biased by food intake of fungi (cheese, mushrooms etc.). For example, Agaricales, a gilled mushroom order (123), was found to be increased in biofilm-positive stool samples. It is likely that this fungus is not in fact involved in biofilm pathogenesis, but rather a fungal food component, which was present in stool samples.

Even though fungal results did not provide significant findings between STs and BFFs or patient cohorts, we did reveal that fungi are a component of polymicrobial biofilms and their composition might be related to the stool mycobiome. What remains unclear, is to what extent fungi are determining factors in biofilm development and how much of their presence in the microbiome is due to nutritional contamination (e.g. cheese, baked goods). To conclude, future studies with bigger sample size and improved methodology could complement these promising but insufficient results and reveal the pathophysiologic role of fungi in intestinal biofilms.

To better study the role of archaea in IBD and IBS, we evaluated their composition in ST and BFFs. We found a high similarity in profiles between biofilms and stool of patients harboring a biofilm (BFF vs. ST-BF-pos), proofing resemblance in the stool microbiome and the matching biofilms. In addition, when comparing archaeal stool composition of patients with and patients without a biofilm (ST-BF-pos versus ST-BF-neg), we detected significant differences. This finding points to a significantly differently structured fecal archaeome in patients who are biofilm-positive compared to biofilm-negative patients. LEfSe analysis revealed *Methanobrevibacter smithii TS146C* to be more abundant in ST-BF-pos than ST-BF-neg.

In microbiome research, archaea are starting to gain recognition as contributing factors to IBD and IBS pathophysiology. For instance, *M. stadtmanae* has been found to be highly abundant and to provoke antibody reaction in IBD patients (68). Examining *M. stadtmanae* in IBS, shows a decreased abundance (71). In addition, *M. smithii* has been shown capable of producing adherence structures to penetrate the intestinal mucosa, a key ability in order to form biofilms (109). Biofilm formation has been shown in vitro for both *M. stadtmanae* and *M. smithii* (110). However, these combined findings have not been demonstrated in the context of gastrointestinal biofilms in IBD and IBS before. In our study, we found *M. smithii* to be enriched in biofilm-positive patients, which taken together with *M. smithii's* known properties of adhesion and biofilm formation, suggests that it may be involved in intestinal biofilm pathogenesis. However, further studies are required to evaluate our results and examine if archaea are drivers of biofilms in IBS and IBD.

Even though archaeal PCR showed quite satisfying results in this study, sequencing revealed only very low richness of archaeal strains. Also, two PCR amplifications and high cycle number (65 in total) could increase unspecific reads or quench low abundant sequences. Furthermore, archaeal diversity was low with only one or two ASVs per sample, indicating that many archaea might not be detected, thereby possibly distorting results. The issue of methodology not only applies to fungi, but also archaea. To better examine and understand the role of archaea in disease context, extraction methods for each sample type need to be optimized and adapted to archaea. Further studies should therefor focus on using better-evaluated methods and bigger sample size.

Despite imperfect sample size and methodology, we showed that biofilm development could be importantly influenced, possibly even triggered by archaea present in the fecal microbiome. As opposed to that, archaea present in biofilms could be released into the gut lumen and impact the fecal archaeome. In both manners, archaea could contribute to biofilm development and subsequently disease pathogenesis of IBS and IBD.

When studying bacterial composition of BFFs, results were quite satisfying. We found significant differences between IBS and UC biofilms, which indicates a disease-specific bacterial signature - a phenomenon described in other studies examining stool samples only. LEfSe analysis further revealed Firmicutes and Eggerthellaceae to be enriched in UC. Here we showed that biofilms too harbor characteristic signatures correlating with disease status. Many studies have examined the bacterial composition of the microbiome in IBD and IBS, showing specific dysbiotic signatures. Specific alterations in the microbiome, like decreased

α diversity, distinct shifts of specific bacterial strains or specific clustering in PCoA, have been shown in IBD and IBS studies (65)(28)(70).

Previously, it has been shown that bacterial biofilms exist in CRC and are closely associated with reduced epithelial integrity and a pro-inflammatory state of the mucosa (50,81). These combined insights indicate that intestinal biofilms might be a common feature in multiple gastrointestinal diseases, including IBS, IBD and CRC, and could be centrally involved in pathophysiology of the intestines. This could imply that gastrointestinal biofilms create a unique milieu, with specific pathophysiologic circumstances, depending on UC or IBS, therefore making it possible for only certain microbes to reside. These circumstances, responsible for which microbes are able to build a biofilm, might be determined by a close interaction of the intestinal mucosa, environmental factors and the microbiome.

Since methodology for bacteria has been developed and optimized over a longer period of time, results are more specific and reliable. Due to well-established DNA extraction methods, sophisticated PCR primers and low cycle number (25 cycles), bacterial results are more robust compared to fungal and archaeal results. In this study, we only checked bacterial content in biofilms, since a closer examination of BFFs and STs was already conducted in another study (112).

In this study, we showed that biofilms have characteristic bacterial signatures depending on disease phenotype. To further evaluate the role of bacteria in biofilms and to analyze microbial signatures more effectively in IBD and IBS, studies with larger cohorts could disguise further specific shifts, perhaps even for fungi and archaea.

A single gastric biofilm was evaluated, since it seems to be a rare feature in the stomach. The biofilm was found in a patient with Barrett's esophagus and examined for bacterial and fungal composition. *Streptococcus*, the most abundant genus in this BFF, is generally a facultative anaerobe, therefore not surviving in environments with oxygen (108). However, its presence is likely due to the biofilm membrane protecting it and creating its own milieu. The presence of fungi in the biofilm highlights the co-existence of different kingdoms within this small ecosystem, or could be due to contamination by fungal DNA in food. All microbes found in the biofilm tend to be found in the oral microbiome, which are usually not present in the stomach.

Only in a study of treatment-resistant *H. pylori* gastritis, gastric biofilms have been suggested before (85). The presence of a biofilm in the stomach shows how well microbes adapt to different milieus, even surviving in extremely acidic environments. By building a stable

outer membrane, microbes within the biofilm are able to survive, which otherwise would not withstand the low pH in the stomach. In a recent study by *Parsons et al*, the gastric microbiome of patients with *H. pylori* gastritis, autoimmune gastritis, *H. pylori* induced atrophic gastritis, patients on proton pump inhibitors and healthy controls were examined. Interestingly, *Streptococcus* was the dominating strain in autoimmune gastritis, which is mirrored in the gastric biofilm of our study. Furthermore, *Veillonellaceae* were also a common feature in autoimmune gastritis, similarly as in our biofilm (124).
Hence, our gastric biofilm shows similar microbial results as in the study by *Parsons et al*, however the composition of our biofilm resembles the microbiome of autoimmune gastritis, which is very unlikely to co-manifest with Barrett's esophagus, since in autoimmune gastritis stomach acidity is absent and Barrett's esophagus is caused by reflux of stomach acid. Therefore, our results stand in contrast to the findings by *Parson et al*. However, information on proton pump inhibitor use of the patient is lacking, which would cause high pH levels comparable to autoimmune gastritis and might allow for a similar stomach milieu.
Nevertheless, the formation of a biofilm in the stomach might be a consequence, if not the escalation of shifts in the gastric microbiome in gastric diseases. However, to evaluate the frequency of gastric biofilms and their resemblance to the gastric microbiome, further studies are needed.

Today both IBD and IBS are seen as multifactorial diseases, with genetics, environmental factors and the microbiome contributing to disease development (1)(34). A central point of action seems to be the intestinal mucosa, where nutritional factors and microbes interact with the epithelial cells. Intestinal barrier function, which forms a physical barrier between the content of the colon lumen and the epithelium, is essential for gut homeostasis. Overlying mucus acts as a first barrier to microbes and keeps them from reaching the epithelial cell line (87).
Certain conditions, like fiber scarcity, can cause some bacterial strains to rely more heavily on mucus for their energy turnover, therefore feeding off intestinal mucus more aggressively. By devouring the protective mucus on top of the epithelial cells, some bacteria advance to the epithelial cell lining and are able to cause inflammatory activation and increased permeability of the barrier (87). In the case of biofilms, a bacterial strain may not only advance to the epithelium, but also succeed to adhere to the intestinal surface. By creating a suitable milieu, other microbes could start to co-inhabit and form a thick layer directly on top of the epithelial cells (39)(91)(92).

This could further cause a toxic micro-environment, interfere with dietary resorption and cause inflammation. However, these circumstances might only lead to disease, if an individual is genetically susceptible, for example by having an attenuated recognition and response to microbial invasion or an easily disrupted intestinal barrier (91). The presence of some, but few biofilms in healthy controls supports this theory. Biofilms might therefore not only occur in disease states but might also serve as an indicator of a dysbiotic microbiome, harming the underlying epithelium.

However, a polymicrobial biofilm could also be a reaction to an already inflammatory mucosa, with microbes being only passively exposed to inflamed tissue. For example, inflammation in IBD might be triggered by internal factors, such as auto-immune like reactions, and the microbiome might only be altered secondary to an existing pro-inflammatory environment (13)(39,40). Triggers to microbes for biofilm development include hostile environments, which are likely to exist in a pro-inflammatory epithelial setting. Nutrient scarcity and antibiotics are examples of a stressing ambience, which might induce bacteria to create biofilms with a protective hydrophobic outer membrane, making them more resistant to hostile immune cells or antibiotics. Furthermore, polymicrobial biofilms might offer more efficient protection by each microbe contributing specific metabolism and defense mechanism to the community, explaining why bacteria, fungi and archaea cooperate in building biofilms (77)(78).

The finding of biofilms is the very first morphological correlate in IBS and might be a key to understand its pathophysiology. As shown in a study by *Wong et al*, high levels of BA are a common feature in IBS-D patients due to malabsorption and increased synthesis (98). The corresponding salts, BS, are known to induce co-aggregation, adhesion to endothelial cells and to trigger biofilm formation in certain bacteria, like *B. fragilis* (99).
These findings could explain how biofilms develop in IBS-D patients and their presence might further explain symptoms like reduced epithelial barrier function and diarrhea due to impaired dietary resorption and micro-inflammation. In addition, biofilms might actually be the often-described small intestinal bacterial overgrowth (SIBO) of IBS patients in the terminal ileum (91), a common location for biofilms. Our hypothesis could link these previous findings in IBS and explain core functions of pathogenesis and pathophysiology of IBS-D.

Shown in a previous study by *Baumgartner et al*, biofilms were mostly found in UC patients when analyzing IBD results (112). This might be explained by UC only affecting the mucosa and submucosa of the colon and sometimes the terminal ileum - the area inhabited by microbes. CD on the other hand is a transmural condition affecting the entire GI tract, expanding further than typical microbiome locations (1). This suggest that a close interaction between the microbiome and intestinal mucosa might be of central importance for UC pathogenesis, but not as much for CD. Biofilms could be an important component of pathogenesis or a manifestation of severe dysbiosis in UC, comprising barrier function loss and inflammation. The less common presence of biofilms in CD might be due to a different mechanism causing inflammation in CD, which might not be as much triggered by microbes but rather internal effects of the immune system; therefore, not typically causing biofilms.

Another interesting result concerns stool and biofilm resemblance. When comparing ST-BF-pos with BFFs, a high similarity was found for archaea and fungi (bacteria were not studied in STs). This suggests that by examining DNA material in stool, biofilm status might be predictable. By using larger studies with a bigger sample number, more specific microbiome constellations could be detected that are linked to biofilm presence. In future studies, microbial stool and biofilm analysis could serve to recognize a specific microbiome composition in the fecal microbiome, which is able to predict biofilm status. This microbial signature could be used as a biomarker for gastrointestinal biofilms, with biofilms themselves serving as an indicator of severe dysbiosis, as it occurs in IBS and IBD, but could also be developed to screen for the diseases themselves. This would permit biofilm detection and screening of the gastrointestinal mucosa for aberrances without requiring colonoscopy - an immense advantage to the health care system and patients who regularly have to undergo an examination.

However, to further evaluate these hypotheses, large cross-sectional studies with bigger cohort groups are needed. Exact differentiation between IBS subtypes and more evenly spread cohorts are required. Also, methodology must advance to detect not only bacteria, but to also grasp fungi and archaea at lower DNA content levels, since both are known to from biofilms in other biological contexts. To improve microbial data and understand pathophysiologic roles of microbes, metagenomic sequencing should be used. In this study, we performed amplicon based sequencing, which depicts all DNA data present. However,

to understand which DNA and therefore which strains are actually active and might play an important role in pathophysiology, transcriptomics would be at an advantage.

In conclusion, macroscopic polymicrobial biofilms are an intriguing and completely novel finding. They point at microbiome dysbiosis of the gut and may be closely involved in IBS and IBD, especially UC, pathogenesis and pathophysiology. In terms of archaea and fungi biofilms tend to resemble their matching stool samples and there is a significant difference between the archaeal composition of stool samples from biofilm-positive and biofilm-negative patients. Improved methodology and larger studies could help develop biomarkers and disguise the role of biofilms in disease context. To completely understand their pathogenic role in the gastrointestinal tract and better evaluate their polymicrobial composition, further studies are needed.

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Concept of project

FB-OSL-04(06)
Konzeptformular für wissenschaftliche Arbeiten

Medizinische Universität Graz

(Arbeits-)Titel *Das Thema der Diplomarbeit ist einem der im Curriculum festgelegten Prüfungsfächer zu entnehmen.*	Detecting the Role of Fungi, Bacteria and Archaea in Intestinal Biofilms of IBS and IBD patients
Untertitel (optional)	
Konzept erstellt von: Erstellt am: Revisionsdatum bei Änderungen: Version: *(Erste eingereichte Version ist „01“)* Matrikelnummer: Studienkennzahl:	Elisabeth Orgler 22.01.2019 01 01433141
Betreuer/in: Institut/Klinik: Kontakt: *(Adresse, Telefonnummer, E-Mail)* Zweite/r Betreuer/in: Kontakt: *(Adresse, Telefonnummer, E-Mail)* MitarbeiterInnen:	Christine Moissl-Eichinger Innere Medizin Auenbruggerplatz 15, 0316 385 72808, christine.moissl-eichinger@medunigraz.at
Kernfrage und Zielsetzung *Wie lautet die Fragestellung?* *Warum ist diese Frage von Bedeutung?* *Welche Ergebnisse sind im Wesentlichen zu erwarten?* *Worin besteht der theoretische Kern der Arbeit?* Sind die Forschungsfrage und die mit dem Projekt angestrebte Zielsetzung für Männer und Frauen gleichermaßen bedeutsam? Detaillierte Angaben!	Die Prävalenzen von Chronisch Entzündlichen Darmerkrankungen (CED) und Reizdarm Syndrom (RDS) steigen kontinuierlich an und die Ätiologie ist nach wie vor ungeklärt. Es handelt sich um multifaktorielle Erkrankungen, bei denen das Mikrobiom (bestehend auf Bakterien, Pilzen, Archaeen und Viren) eine entscheidende Rolle zu spielen scheint. Biofilme der Darmschleimhaut wurden bereits beim Coloncarcinom entdeckt und als relevant für die Pathogenese anerkannt. In dieser Arbeit werden endoskopisch sichtbare Biofilme bei CED und RDS untersucht. Die zentrale Forschungsfrage beschäftigt sich mit dem Erkennen von Mikroben (Pilze, Bakterien und Archaeen) und dem Verstehen ihrer Rollen im Krankheitskontext. Dafür werden Biofilme während der Koloskopie von RDS-, CED - Patienten und Kontrollen gewonnen. Anschließend wird DNA extrahiert und diese mittels PCR und darauffolgendem Next Generation Sequencing auf Pilze, Bakterien und Archaeen getestet. Das Ziel der Arbeit ist Unterschiede in den Biofilmen und Stuhlproben von gesunden Kontrollpersonen und erkrankten RDS/ CED Patienten zu erkennen. Durch die Ergebnisse wird ein besseres Verstehen der Ätiologie der beiden Erkrankungen erhofft. Die Resultate sollen auch in Form einer Publikation veröffentlicht werden. Die Forschungsfragen sind für Männer und Frauen gleichermaßen bedeutsam.

Kurzbeschreibung (max. 20 Zeilen) *Worin besteht der Neuigkeitswert?*	Intestinale Biofilme wurden bei RDS noch gar nicht und bei CED kaum beschrieben. Außerdem wurden Biofilme im Darm bisher nur auf Bakterien, nicht aber auf Pilze oder Archaeen untersucht. Im Kontext anderer Krankheiten (z.B. Zahnplaques, Gelenksprothesen) wurden jedoch aus verschiedenen Spezies bestehende Biofilme beschrieben. In dieser Arbeit werden intestinale Biofilme erstmals auf Pilze und Archaeen bei CED und RDS Patienten untersucht und die Ergebnisse mit Kontrollen verglichen. Ebenfalls werden Stuhlproben derselben Studienteilnehmer, sowie weiterer Kontrollen auf Pilze, Archaeen und Bakterien getestet.
Methodenwahl *Welche Methoden stehen zur Beantwortung der Frage zur Verfügung? Wieso wählen Sie genau diese Methode?*	DNA Extraktion, PCR, Next Generation Sequencing
Ethikkommissionsvotum *Ist ein Votum der Ethikkommission erforderlich?* *Siehe Informationsblatt „Genehmigung Ethikkommission“*	☐ Erforderlich ☐ Nicht erforderlich x☐ Bereits vorhanden
Datenerhebung (falls zutreffend) *Werden aufgrund der oben genannten Methodenwahl medizinische Daten benötigt? Wenn ja, welche?* *Mit welcher Fallzahl ist zu rechnen? Wie wurde die Fallzahl ermittelt?* *Wie ist das PatientInnenkollektiv zu beschreiben (Mindest-/Höchstalter, Geschlechtsverteilung, Begleiterkrankungen, etc.)?* *Bitte beachten Sie, dass eine Weitergabe der Daten an projektfremde Personen gemäß Datenschutzgesetz nicht zulässig ist. Das Bekanntwerden von PatientInnendaten ist durch Pseudonymisierung (Codierung mit fortlaufender Nummer) und ggf. Zugriffsbeschränkungen zu verhindern.*	Nicht anwendbar, medizinische Daten werden nicht benötigt.
Datenauswertung *Welche Hauptzielgröße wird analysiert (z.B. Alter bei Diagnosestellung/Alter bei Operation/Diagnose, etc.)? Wie wird die Hauptzielgröße analysiert?* *Welche Nebenzielparameter sollen betrachtet werden? Mit welchen Methoden erfolgt die Auswertung?*	Das Ziel ist das Erkennen von Trends im Mikrobiom von RDS- und CED – Patienten und diese mit Kontrollen zu vergleichen. Ein besonderer Fokus liegt hier im Erkennen von Unterschieden beim Mykobiom.
Zeitplan (grob strukturiert) *Wann wird mit der Arbeit begonnen? Wann wird ein Antrag bei der Ethikkommission gestellt, sofern ein Votum erforderlich ist?* *Welche Meilensteine wurden zwischen dem/der Studierenden und den BetreuerInnen vereinbart?* *Wann ist voraussichtlich mit der Beendigung der Arbeit zu rechnen?* *Welche formalen Schritte sind für die Umsetzung der Diplomarbeit notwendig?*	Beginn der Arbeit: 01.09.2018. Voraussichtliches Ende: 31.06.2019 Das Projekt wird in Kooperation mit der Forschungsgruppe um Prof. Christoph Gasche von der Medizinischen Universität Wien durchgeführt. Die Versuche, sowie die Proben stammen vom AKH Wien und das Projekt wird vor Ort von Prof. Gasche/ Dipl.-Ing. Maximilian Baumgartner mitbetreut. Das Verfassen der Arbeit erfolgt in Graz und die Betreuung wird von Prof. Christine Moissl-Eichinger gewährleistet.
Referenzen Welche Literatur ist relevant? Gibt es Vergleichsstudien?	Dejea CM, Fathi P, Craig JM, et al. Patients with familial adenomatous polyposis harbor colonic biofilms containing tumorigenic bacteria. Science. 2018;359(6375):592-597. Swidsinski A, Weber J, Loening-baucke V, Hale LP, Lochs H. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. J Clin Microbiol. 2005;43(7):3380-9. Sokol H, Leducq V, Aschard H, et al. Fungal microbiota dysbiosis in IBD. Gut. 2017;66(6):1039-1048. Peleg AY, Hogan DA, Mylonakis E. Medically important bacterial-fungal interactions. Nat Rev Microbiol. 2010;8(5):340-9.

	Fanning S, Mitchell AP. Fungal biofilms. PLoS Pathog. 2012;8(4):e1002585. Sam QH, Chang MW, Chai LY. The Fungal Mycobiome and Its Interaction with Gut Bacteria in the Host. Int J Mol Sci. 2017;18(2) Nash AK, Auchtung TA, Wong MC, et al. The gut mycobiome of the Human Microbiome Project healthy cohort. Microbiome. 2017;5(1):153.
Benötigte Ressourcen *Werden Geld- oder Sachmittel von Einrichtungen der Med Uni Graz benötigt?* Die Vergabe ist nur zulässig, wenn die Leiterin/der Leiter dieser Einrichtung über die beabsichtigte Vergabe informiert wurde und diese nicht binnen eines Monats untersagt hat.	Nein

Supplements

Supplement 1. Extraction and Purification Procedure for RNA and DNA.

Method adapted from: Griffiths, R.I., Whiteley, A.S., O'Donnell, A.G., and Bailey, M.J. (2000) Rapid method for co-extraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. Appl Environ Microbiol 66: 5488-5491.

All solutions and glassware should be treated with diethyl pyrocarbonate (DEPC) prior to use.

Nucleic acid extraction

Cool down the centrifuge to 4°C

Label one bead-beating tube (Bio-101 Multimix 2 Matrix tube) and three 2ml tubes per sample.

Add 500µl of CTAB extraction buffer and 500µl phenol-chloroform-isoamyl alcohol (25:24:1; pH 8.0) to the sample and transfer to the bead beating tube.

Bead-beat for 30 seconds at 5.5m/s.

Centrifuge at 13,000 rpm for 5 minutes at 4°C and transfer upper, aqueous phase into a new 2ml tube.

Add a further 500 µl of CTAB extraction buffer and 500 µl phenol-chloroform-isoamyl alcohol (25:24:1; pH 8.0) to the bead beating tube and lyse for 30 seconds at 5,5 m/s in the bead beater.

Centrifuge at 13,000 rpm for 5 minutes at 4°C and combine the aqueous phase with that previously collected.

Add 1 volume (1ml) of chisam (chloroform-isoamyl alcohol 24:1) and spin at 13,000rpm for 5 minutes at 4°C.

Transfer upper, aqueous phase into a new 2ml tube and repeat chisam wash step to remove residual phenol.

Add: 0.1 volume (100µl) 3M Na-Acetate and 0.6 volume ice-cold isopropanol (600µl) and mix by inversion.

Precipitate nucleic acids for at least 2 hours at room temperature.

Centrifuge at 13,000rpm for 10 minutes at 4°C, remove supernatant.

Wash the pelleted nucleic acids in 500 µl of ice cold 70% ethanol, centrifuge at 14,000 rpm for 10 minutes at 4°C and remove supernatant.

Dry nucleic acids in vacuum centrifuge for 3 minutes at 30^0C (without spinning).
Resuspend the pellet in 50µl of DEPC treated MilliQ water and place on shaking incubator for 20 minutes at 30^0C to facilitate dissolving of the pellet.
Calculate quantity of nucleic acids extracted using the NanoDrop spectrophotometer and run (<500ng) of extracted nucleic acids on a 1% agarose gel for 30 minutes at 100V.
Store extracted nucleic acids at -80^0C.

Column purification of DNA and RNA
Purifications performed using the Qiagen Allprep DNA/RNA mini kit.
Kit recommends using a maximum of:
30mg of fresh or frozen tissue
Not loading more than 20µg of DNA into the column.

Suspend extracted nucleic acid (40mg) in 600µl of RLT plus buffer and load into an Allprep spin column.
Centrifuge for 30 seconds at 14,000 rpm.
Place the AllPrep DNA spin column in a new 2ml collection tube and store at 4ºC for DNA purification.
Use the flow-through for RNA purification.

Genomic DNA purification
Add 500µl of Buffer AW1 to the AllPrep DNA spin column, centrifuge for 15 seconds at 14,000 rpm and discard the flow-through.
Add 500µl of Buffer AW2 to the AllPrep DNA spin column, centrifuge for 1 minute at 14,000 rpm and discard the flow-through.
Discard flowthrough and centrifuge for 1 minute at 14,000 rpm to dry the spin column membrane.
Place the AllPrep DNA spin column in a new 1.5ml collection tube. Add 100µl Buffer EB (65^0C) directly to the spin column membrane and incubate at room temperature for 1 minute.
Centrifuge for 1 minute 14,000 rpm to elute DNA.
Repeat step 5 to elute further DNA.

Reagents
CTAB extraction buffer

Ingredient	Quantity
10% CTAB (wt/vol) in 0.7M NaCL	50ml
240mM potassium phosphate (KH_2PO_4)	50ml
DEPC	100µl

Combine ingredients (except DEPC) into a clean Schott bottle and adjust to pH 8.0.

Add DEPC in the fume hood and stir overnight.

Autoclave to sterilise and deactivate DEPC.

DEPC treated milliQ water

add 0.1% (v/v) DEPC to solution/water

shake for 1h to O/N on orbital shaker OR 20-30min with a magnetic stirrer

DEPC must then be completely destroyed by autoclaving (15-45min at 15psi on liquid cycle)

If the fruity smell is faint, autoclaving was sufficient. Even after a long 45min autoclave treatment the smell will not be completely gone. DEPC in unstable in aqueous solutions. It hydrolyses quickly to CO_2 and ethanol. Half-life in phosphate buffer at pH 6 is 20min.

DEPC cannot be used with solutions that contain nucleophiles such as Tris (amines) and HEPES (amines) buffers or mercaptans (thiol). In such cases, use DEPC-treated water to generate the solution.

Although H_2O prepared with reverse osmosis systems like Milli-Q is free of RNases (Huang 1995), it can be contaminated by microbial growth if the machine and piping are not well maintained. This is especially problematic in centralised water systems with metres of piping.

The fruity smell coming from ageing DEPC-containing solutions stems from the esters that are generated when ethanol produced from hydrolysis reacts with residual carboxylic esters. It is normal that some fruity smell lingers after DEPC removal and is not necessarily a sign of incomplete DEPC hydrolysis.

Traces of DEPC modify purine residues (A+G) in RNA by carboxymethylation. Therefore, DEPC must always be removed from solutions or containers by autoclaving or heating at 100°C for 15 min. Cell-free translation of carboxymethylated RNA will yield less protein than with unmodified template. Hybridisation is not seriously affected unless the RNA probe is heavily carboxymethylated.

DEPC is carcinogenic (it carboxymethylates purines) and should be handled with care. Wear gloves!

Supplement 2. IHMS DNA extraction protocol Q.

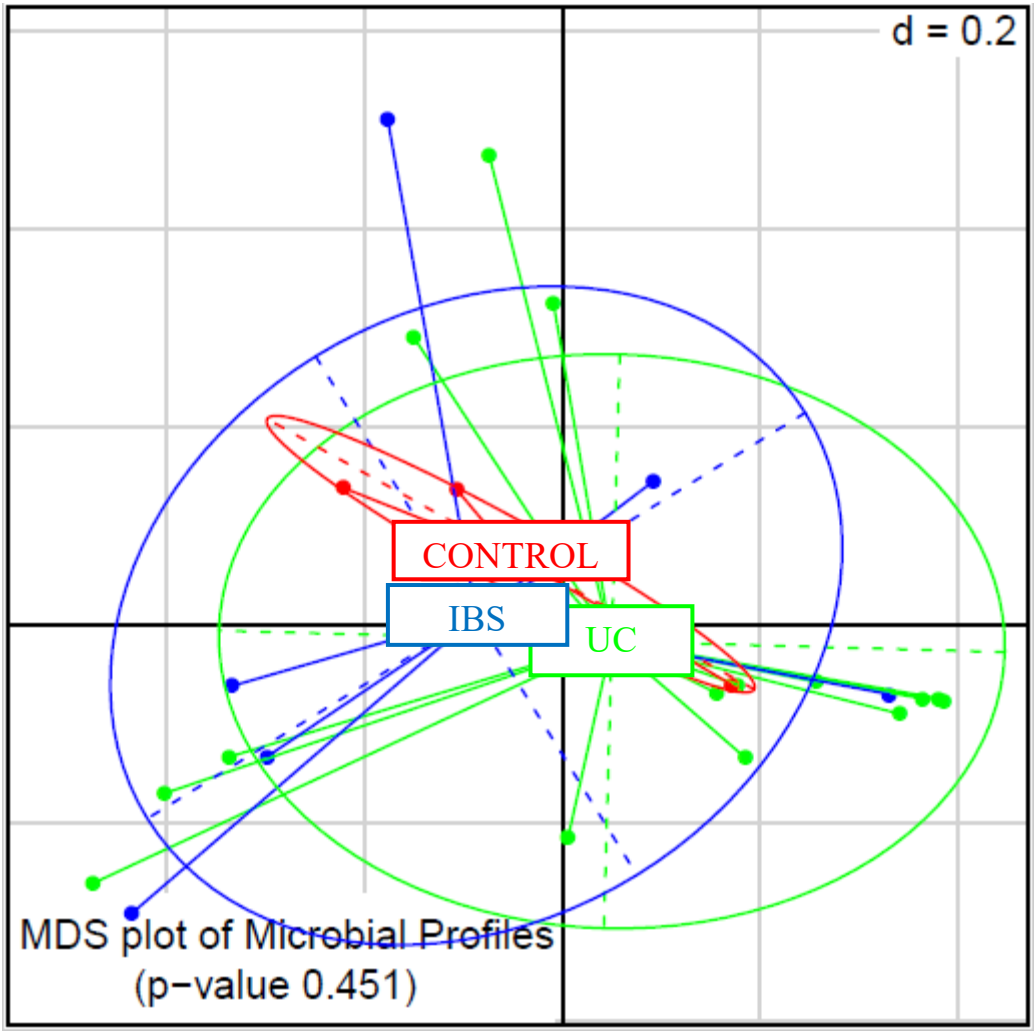
Fecal DNA extraction with the use of Qiagen QIAamp DNA stool kit

1. Homogenize the 150 to 200mg frozen feces with 1.0mL ASL lysis buffer of the kit by vortexing for 2 min in a 2mL tube containing 0.3g of sterile zirconia beads Ø 0,1mm zirconia (BioSpec, Cat. No. 11079101z). [if buffer shows precipitate, heat at 70°C before use]
2. Incubate for 15min at 95°C.
3. Cells are mechanically lysed by running the Fastprep™ Instrument for 1 min of bead beating at 6500 rpm and resting 5 min. Repeat 7 times.
4. Samples are allowed to cool down on ice for 2 min.
5. Samples are centrifuged at 16000 x g, 4°C, for 5 min.
6. Supernatant is transferred to a new 2mL tube.
7. The pellet is mixed with 300µL ASL lysis buffer of the kit, and steps 2-5 are repeated.
8. Supernatants are pooled in the new 2mL tube.
9. Add 260µl of 10M ammonium acetate to each lysate tube, mix well, and incubate on ice for 5 min.
10. Centrifuge at 16000 g, 4°C, for 10 min.
11. Transfer the supernatant to two 1.5mL Eppendorf tubes, add one volume of isopropanol, mix well, and incubate on ice over night.
12. Centrifuge at 16000 g, 4°C, 15min, remove the supernatant using aspiration, wash nucleic acids pellet with 70 % EtOH (0,5mL) and dry the pellet under vacuum for 3 min.
13. Dissolve the nucleic acid pellet in 100µL of AE-Buffer and pool the two aliquots.
14. Add 2µL of DNase-free RNase (10mg/mL) and incubate at 37°C, 15 min.
15. Add 15µL proteinase K and 200µL AL buffer to the supernatant, vortex for 15 sec and incubate at 70°C for 10 min.
16. Add 200µL of ethanol (96-100%) to the lysate, and mix by vortexing.
17. Transfer to a QIAamp spin column and centrifuge at 16000 g for 1 min, at Room Temperature (RT).
18. Discard flow through, add 500µL buffer AW1 (Qiagen) and centrifuge at 16000 g for 1 min, at RT.
19. Discard flow through, add 500µL buffer AW2 (Qiagen) and centrifuge at 16000 g for 3 min, at RT
20. Dry the column by centrifugation at RT for 3.5 min.
21. Add 200µL Buffer AE (Qiagen), incubate for 2 min at RT
22. Centrifuge for 1 min at 16000 g to elute DNA.

Quality control: use 1% agarose gel

Sample concentration: use Nanodrop or Qubit

Supplement 3. Fungal composition of BFFs: UC vs. IBS vs. controls.



Supplement 4. Most abundant fungal DNA reads.

ASV1
TCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGTTTAGGAAAGGGGGCAACCTCATTCTGGAACCGAGAAGCTG
GTCAAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGATTTGCTTAATTGCA
CCACATGTGTTTTTCTTTGAAACAAACTTGCTTTGGCGGTGGGCCCAGCCTGCCGCCAGAGGTCTAAACTTACAACCAATTTTTTATCAACTT
GTCACACCAGATTATTACTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCAT

ASV3
CCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGTTTAGGAAAGGGGGCAACCTCATTCTGGAACCGAGAAGCTG
GTCAAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGATTTGCTTAATTGCA
CCACATGTGTTTTTCTTTGAAACAAACTTGCTTTGGCGGTGGGCCCAGCCTGCCGCCAGAGGTCTAAACTTACAACCAATTTTTTATCAACTT
GTCACACCAGATTATTACTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCAT

ASV29
TCGCCCGTCGCTACTACCGATTGAATGGCTCGGTGAGGCCTTCGGACTGGCCCAGGGAGGTCGGCAACGACCACCCAGGGCCGGAAAGTTG
GTCAAACCCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGTCTA
ACCACCGGGATGTTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAA
ACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGCAT

ASV12
TCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCTTCCGGATTGATTAACTGGAGAGGGCAACTTTTCTGGTTGAACGAGAAGCTAG
TCAAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATGAATTAATAATATTTGTG
AAATTTCAACAAACAACATCAATTTTATAGTCTATTATTTTTAATTAAAACTTTTAACAATGGATCTCTTGGTTCTCGTAT

ASV7
TCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTG
GTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCCCTCT
GGGTCCAACCTCCCACCCGTGTTTATTTACCTTATTGCTTCGGCGGGCCCGCCTTAACTGGCCGCCGGGGGGTTTACACCCCCGGGCCCGCG
CCCGCCGAAGACACCCCGAACTCTGTCTGAAGAATGCAGTCTGAGAACAAATATAAATTATTTAAAACTTTCAACAACGGATCTCTTGGTTC
CGGCAT

ASV9
TCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCTTCCGGATTGATTAATTGGAGAGGGCAACTTTTCTGATTGAACGAGAAGCTAG
TCAAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATGAATTAATAATATTTGTG
AAATTTCAACAAACAACATCAATTTTATAGTCTATTATTTTTAATTAAAACTTTTAACAATGGATCTCTTGGTTCTCGTAT

ASV2
TCGCCCGTCGCACCTACCGATTGAATGGTCCGATGAACACTTTGGATTTTAGTAATGTCAGTCTTAACGGATTGATGATTATTGAGAGAAGT
TGTGTAAATCTTACCATTTAGAGGAAGGTGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATAGAATGCCGTAAGG
CTTAGTAGCCTGTTGGTATATCCAATGGGTATTTGTTGTAATTATGTTGTAAAACATAGTTATAATGAATTAGGTGATGGTAGTGAAAACAA
CTATATGCGATGGATATCTTGGCTCTTGATT

ASV48
TCGCCCGTCGCTCCTACCGATTGAACGGTCCGGCGAGAACTCCGGATGGTCGGCACGCAGGGGTCAAACCCTGTGTCCGTGCCAGAAGCTG
TTCGAACCTTACCGTTTAGAGGAAGGAGAAGTCGTAACAAGGTATCCGTAGGTGAACCTGCGGATGGATCATTTGAAGAACGTCGTCCATC
GGTCGTGAACTTCCCTTCCTCCGGGAGGGGAAACGTCACGCTCCGACACCTGATAGCGAACGGTCGAAGTCCCTTCCCGGGACCTCAAGGA
CTCAGGTCCTCGAATCCATCCCGGGGAGACTCCACAACCGTAGTCCCCTCGGCGAAACATCGATCGAGGGAGGGATCGCCAGTCGATCCTT
CCGAGAAACGAGAATAACCTAGTCGACTACTTTCGACGACGGATACCTCGGTTCCCACGC

ASV4
TCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCTTCCGGATTGATTAACTGGAGAGGGCAACTTTTCTGGTTGAACGAGAAGCTAG
TCAAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATGAATTATAAATATTTGTG
AATTTACCACAGCAAACATCAATCATACAATCAATAATTAAAAAATTAAAACTTTTAACAATGGATCTCTTGGTTCTCGTAT

ASV17
TCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGACCTTCGGATTGGTGCTTGGTGGCCGCAAGGCTGCTGAATGCTGAAAAGTTGGTC
AAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAACAAATCATTTGGTGCTGGA
TTTATCCAGCACTATATTAACCCGAACCTGTGCCTTTTAGGCCAGGCTAGGTGTGGGTGTATAGTTCTCCTTCGGGGGAGCTGTGCATTCGCG
GTGAAGACGCAAGTCGGATCCACATAGACGCTTGTTTTTGTTTGACAGATTTCAATTGTATCCAAAATTAAATGACAACTTTTAACAATGGA
TCTCTTGGCTCTTGCAA

ASV89
TCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGCTTAAGAAAGGGGGCAACCTCATTCTGGAGCTGAAAAGCTG
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TACACATGTTTTTTTAGAGAACTTGCTTGCAAGAACACTAATAATTTACTTAGTCAACAAATAAAAATATCAAAACTTTCAACAACGGATCT
CTTGGTTCTCGCAT

ASV5
CCGCCCGTCGCACCTACCGATTGAATGGTCCGATGAACACTTTGGATTTTAGTAATGTCAGTCTTAACGGATTGATGATTATTGAGAGAAGT
TGTGTAAATCTTACCATTTAGAGGAAGGTGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATAGAATGCCGTAAGG
CTTAGTAGCCTGTTGGTATATCCAATGGGTATTTGTTGTAATTATGTTGTAAAACATAGTTATAATGAATTAGGTGATGGTAGTGAAAACAA
CTATATGCGATGGATATCTTGGCTCTTGATT

ASV13
CCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGTTTAGGAAAGGGGGCAACTCCATTCTGGAACCGAGAAGCTG
GTCAAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGATTTGCTTAATTGCA
CCACATGTGTTTTTCTTTGAAACAAACTTGCTTTGGCGGTGGGCCCAGCCTGCCGCCAGAGGTCTAAACTTACAACCAATTTTTTATCAACTT
GTCACACCAGATTATTACTTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCAT

ASV100
TCGCCCGTCGCTACTACCGATTGAATGGCTCGGTGAGGCCTTCGGACTGGCTCCAGGGGGTTGGCAACGACCCCCCAGAGCCGGAAAGTTG
GTCAAACCCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGCGGGCCCTCT
GGGTCCAACCTCCCATCCGTGTCTATCTGTACCCTGTTGCTTCGGCGTGGCCACGGCCCGCCGGAGACTAACATTTGAACGCTGTCTGAAGT
TTGCAGTCTGAGTTTTTAGTTAAACAATCGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCAT

ASV39
CCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGACCTTCGGATTGGTGCTTGGTGGCCGCAAGGCTGCTGAATGCTGAAAAGTTGGTC
AAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAACAAATCATTTGGTGCTGGA
TTTATCCAGCACTATATTAACCCGAACCTGTGCCTTTTAGGCCAGGCTAGGTGTGGGTGTATAGTTCTCCTTCGGGGGAGCTGTGCATTCGCG
GTGAAGACGCAAGTCGGATCCACATAGACGCTTGTTTTTGTTTGACAGATTTCAATTGTATCCAAAATTAAATGACAACTTTTAACAATGGA
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ASV75
TCGCCCGTCGCTCCTACCGATTGAATGGTCCGGTGAAGTGTTCGGATCGAGGCGACGGGGGCGGTTCGCCGCCCGCGACGTCGCGAGAAGT
CCACTGAACCTTATCATTTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCCACTGAC
GAGGACGACCGTGAATGCGTCAACGATTGCTCGTCGGGCTCGTCCCGACAACACCCCGAATGTCGGTTCGCCCTCGGGCGGGACGATCGAG
GGGATGAACTACCAACCCCGGCGCGGATAGCGCCAAGGAACACGAACATCGAAGTCGGAGGGCCTCGCTGCATGCAGGAGGCTACAATTC
CGACGGTGACCCCATTGGACGACTCTCGGCAACGGATATCTCGGCTCTCGCAT

ASV80
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CAGAACGACCTGCTAACTCGTAAACACATTGGGCAAGCGTCGGGGGGCTTTTGTCCCTTGTTCGCGAATCCCTGGTAGGTGGCCCCTCCTGG
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ASV22
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ASV31
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GGGTCCAACCTCCCATCCGTGTCTATCTGTACCCTGTTGCTTCGGCGTGGCCACGGCCCGCCGAAGACTAACATTTGAACACTGTCTGAAGT
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ASV108
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CAGAACGACCCGCGAACTCGTTTTAAACACCGGGGGCGGCGCTCGCTCGTCGCGCGCCTCCCCCCGTCGCCCGAGGCGCGCAAGCTCTTCG
GGCGACCAACGAACCCCGGCGCGGAAAGCGCCAAGGAATACTACAATCGACAGCCCTCCCCCTCGCGCCCCGTTCGCGGATCGTGCGGGG
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ASV37
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CATACGGACGCCACAAAGTGTCCCTGGCCGCCTACACCCACTATACATCCACAAACCCGTGTGCACTGTCCTTGGAAAGGGCATTTGGAGA
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AACTCGAATGGTTAGTATGAACGTGGAACTTGGTTGGACCGTCACTGGCCAACAAACTATACACAACTTTCGACAACGGATCTCTTGGTTCT
CCCAT

ASV11
TCGCCCGTCGCTACTACCGATTGGATGAGTGGGTGAGGAGGTGGGGGGGGACTGAGAGATTGGTCCTGAACACGGCCAAACCCGTTCATCTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCAGTGTGGGGAGAGAGGGGGAGATGGGACTGAGGTCTCG
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ASV20
CCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCTTCCGGATTGATTAATTGGAGAGGGCAACTTTTCTGATTGAACGAGAAGCTAG
TCAAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATGAATTAATAATATTTGTG
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ASV107
TCTCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGTTTAGGAAAGGGGGCAACCTCATTCTGGAACCGAGAAGCTG
GTCAAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGATTTGCTTAATTGCA
CCACATGTGTTTTTCTTTGAAACAAACTTGCTTTGGCGGTGGGCCCAGCCTGCCGCCAGAGGTCTAAACTTACAACCAATTTTTTATCAACTT
GTCACACCAGATTATTACTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCAT

ASV21
CCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGTTTAGGAAAGGGGGCAACTCCATTCTGGAACCGAGAAGCTG
GTCAAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGATTTGCTTAATTGCA
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ASV6
TCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGTTTAGGAAAGGGGGCAACTCCATTCTGGAACCGAGAAGCTG
GTCAAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGATTTGCTTAATTGCA
CCACATGTGTTTTTCTTTGAAACAAACTTGCTTTGGCGGTGGGCCCAGCCTGCCGCCAGAGGTCTAAACTTACAACCAATTTTTTATCAACTT
GTCACACCAGATTATTACTTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCAT

ASV77
TCGCCCGTCGCTACTACCGATTGAATGGCTCGGTGAGGCCTTCGGACTGGCCCAGGGAGGTCGGCAACGACCACCCAGGGCCGGAAAGTTG
GTCAAACCCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACAAGAACGCCCGGGCTT
CGGCCTGGTTATTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAA
CTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGCAT

ASV55
CCGCCCGTCGCTACTACCGATTGAATGGCTCGGTGAGGCCTTCGGACTGGCCCAGGGAGGTCGGCAACGACCACCCAGGGCCGGAAAGTTG
GTCAAACCCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGTCTA
ACCACCGGGATGTTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAA
ACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGCAT

ASV194
TCGCCCGTCGCTACTACCGATTGAATGGCTCGGTGAGGCCTCCGGACTGGCTCAGGAGGGTTGGCAACGACCCCCCAGAGCCGGAAAGCTG
GTCAAACCCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGTTTCTC
CGAAGCCCAACCTCCCACCCGTGTATACCGTACCCTGTTGCTTCGGCGGGCCCGCCTCACGGCCGCCGGGGGGCCTCGCGCCCCCGGGCCCG
CGCCCGCCGAAGACCCCAACACGAACACTGTCTGAAAAGTGCAGTCTGAGTCGATTGTTACCAATCAGTCAAAACTTTCAACAATGGATCT
CTTGGTTCCGGCAT

ASV177
TCGCCCGTCGCTCCTACCGATTGAATGGTCCGGTGAAGTGTTCGGATCGTGGCGACGTGGGCGGTTCGCTGCCTGCGACGTCGCGAGAAGTC
CACTGAACCTTATCATTTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTAAACATC
AAACGACCCGTGAACGTGTTTTCAAACCTTTTGTGTCGGGGGGAGCATTCGTGCCCCCTTTGATGCCTAAACCAAAACCGGCGCAAGTCGCG
CCAAGGAGCTCAAACGAATAAGCTTGCCCCTTGCCCCGGTCTCGGTGTGCGGGGGGCAAAGCATTCTTGTCGTATTATTCACAACGACTCTC
GGCAACGGATATCTCGGCTCTCGCAT

Supplement 5. Archaeal composition of STs: UC vs. IBS vs. controls.