Universitat Autònoma de Barcelona

Observational study in Ulcerative Colitis to investigate the composition of the intestinal microbiota in patients in long-term remission

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Observational study in Ulcerative Colitis to investigate the composition of the intestinal microbiota in patients in long-term remission

A doctoral thesis presented by **Claudia Herrera de Guise** to aim for the degree of Doctor

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LIST OF ABBREVIATIONS

16S rRNA, 16S ribosomal RNA

AMPs, antimicrobial peptides

APCs, antigen presenting cells

CD, Crohn's disease

CRP, C reactive protein

DCs, dendritic cells

DGGE, denaturing gradient gel electrophoresis

ELISA, enzyme-linked immunosorbent assay

FDR, false discovery rate

FISH, fluorescence in situ hybridization

FMT, fecal microbiota transplantation

GI, gastrointestinal

HC, Healthy controls

IBD, inflammatory bowel disease

IFN-γ, interferon gamma

IL, interleukin

ITS, internal transcribed spacer

MAMPs, microbe-associated molecular patterns

OTUs, operational taxonomic units

LIST OF ABBREVIATIONS

PAMPs, pathogen associated molecular patterns

PCoA, principal coordinate analysis

PCR, polymerase chain reaction

qPCR, quantitative PCR

rDNA, deoxyribonucleic acid recombinant

SCFA, short chain fatty acids

SCCAI, Simple Clinical Colitis Activity Index

TGGE, temperature gradient gel electrophoresis

Th, T-helper cells

TLRs, toll like receptors

TNF-a, tumour necrosis factor alpha

Treg, T-regulatory cells

UC, ulcerative colitis

UC fl, ulcerative colitis flare

UC lr, ulcerative colitis long remission

UC sr, ulcerative colitis short remission

UPGM, unweighted pair group method with arithmetic mean

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SUMMARY

Ulcerative colitis (UC) is a chronic idiopathic inflammatory disease that affects the large bowel. It is one of the two major disorders under the broad term of Inflammatory Bowel Disease (IBD). The etiology of UC involves an immune response to an imbalanced gut microbiota in genetically susceptible individuals, following unknown triggering events. Microbiome studies have found a lower microbial richness and diversity in the gut microbiota of IBD patients than healthy controls, and the drop is especially manifested during and after flares of disease. The gut microbiome is essential in maintaining health and mediating illness, and environmental and host factors influence its composition. Clinical, endoscopic, and histological remission positively impacts the natural history of UC. Reaching these goals impacts the natural history of UC.

This thesis aimed to determine if UC patients who reach clinical, endoscopic, and histological remission for an extended period would present a different gut microbiota composition than UC patients with shorter remission lengths or active disease. UC patients in long remission will show a gut microbiota that is similar to healthy individuals.

For this purpose, we analyzed fecal samples of UC patients in long remission, UC patients in short remission and UC patients in flare and compared it to healthy individuals. We used two different methods: 16S rRNA gene sequencing and qPCR for specific bacteria. We also sought to explore non-bacterial constituents of the gut microbiota by determining the fungal load using qPCR.

As expected, we observed dysbiosis in UC with a reduction in diversity and richness, underrepresentation of beneficial bacteria, and the gain of potentially harmful microbes. This dysbiosis was greater in UC patients in disease-flare but was also present in UC patients in short remission. UC patients who were able to achieve a long-term, stable, deep, and histological remission of their disease presented a gut bacterial composition closer to health, thus less dysbiotic. Beyond the bacterial component, we found a link between fungi abundance and inflammatory status in UC. Patients in disease-flare present a greater abundance of the fungal load than patients in remission, but these

results must be interpreted cautiously and further evaluated in larger, longitudinal studies. We believe that a 'healthier' gut microbiota in UC patients could potentially be a goal to pursue in order to define disease remission further.

RESUM

La colitis ulcerosa (CU) és una malaltia inflamatòria idiopàtica crònica que afecta l'intestí gros. És un dels dos trastorns principals que engloba el terme Malaltia Inflamatòria Intestinal (MII). L'etiologia de la CU involucra una resposta immune a una microbiota intestinal desequilibrada en individus genèticament susceptibles, després d'esdeveniments desencadenants desconeguts. Els estudis de la microbiota han trobat una menor riquesa i diversitat en la microbiota intestinal en els pacients amb MII que en els controls sans, i aquesta descans de la diversitat es manifesta especialment durant i després dels brots de la malaltia. El microbioma intestinal és essencial per a mantenir la salut; els factors ambientals i de l'hoste influeixen en la composició de la microbiota intestinal. La remissió clínica, endoscòpica i histològica impacta positivament en la història natural de la CU. Aconseguir aquests objectius de remissió impacta en la història natural de la CU.

Aquesta tesi té com a objectiu determinar si els pacients amb CU que aconsegueixen la remissió clínica, endoscòpica i histològica durant un període prolongat presenten una composició de microbiota intestinal significativament diferent a la dels pacients amb CU en períodes de remissió més curts o amb malaltia activa. És a dir si els pacients amb CU en remissió perllongada mostren una microbiota intestinal similar a la dels individus sans. Per a això, hem analitzat mostres fecals de pacients amb CU en remissió llarga, pacients amb CU en remissió curta i pacients amb CU en brot i la hem comparat amb les mostres d'individus sans. Hem utilitzat dos mètodes diferents: seqüenciació del gen 16S ARNr i qPCR per a bacteris específics. També hem explorat els components no bacterians de la microbiota intestinal mitjançant la determinació de la càrrega fúngica mitjançant qPCR. Com es preveia, observem la presència de disbiosis en la CU amb una reducció en la diversitat i riquesa bacterianes, sub-representació de bacteris beneficiosos i l'increment de bacteris potencialment nocius. Aquesta disbiosis és més pronunciada en pacients amb CU en brot de la malaltia, però també és present en pacients amb CU en remissió curta. Els pacients amb CU que van aconseguir una remissió profunda i histològica estable i a llarg termini de la seva malaltia, presenten una composició bacteriana intestinal més pròxima a la dels controls sans, per tant menys "disbiótica". Més enllà del component bacterià, també trobem un vincle entre l'abundància de fongs i l'estat

inflamatori en la CU. Els pacients en brot de la malaltia presenten una major abundància de càrrega fúngica que els pacients en remissió, però aquests resultats han d'interpretar-se amb cautela i avaluar-se més a fons en estudis longitudinals més amplis. Creiem que una microbiota en els pacients amb CU semblant a la que trobem en individus “sans”, pot ser d’utilitat per a definir amb més precisió la remissió de la malaltia.

1.1 Ulcerative colitis

Ulcerative colitis (UC) is a chronic idiopathic inflammatory disease that affects the large bowel. It is one of the two major disorders under the broad term of inflammatory bowel disease (IBD), which also includes Crohn's disease (CD). The first subtype of IBD characterized as a distinct entity was UC. In 1859, Dr. Samuel Wilks first described UC when he wrote on "idiopathic colitis" and recognized it as a distinct entity from the then more common bacillary dysentery (1). At the beginning of the 20th century, Hawkins described the chronic and relapsing nature of the disease course. Sir Arthur Hurst gave a complete description of UC, including its sigmoidoscopic appearance and differentiation from bacillary dysentery. In 1909 the Royal Society of Medicine in London held a symposium where over 300 cases of UC collected from London hospitals were presented and discussed (2). The etiology of UC remained elusive, and an infectious or psychosomatic origin was considered its primary cause. It would not be until the latter half of the century that genetic research and, in the last several decades, immunology would take the central stage of research to unravel the pathogenesis of UC. Based on the progress made through many approaches in recent years, it is increasingly evident that a critical pathogenic mechanism underlying IBD development is the loss of tolerance and dysregulation of the immune response to commensal intestinal microorganisms in genetically susceptible individuals. Thus, the pathogenesis of this complex disease requires the interaction of host genetics, immune dysregulation, internal microenvironment (the gut microbiome), and the external environment.

1.1.1. Epidemiology

The worldwide incidence of IBD increased dramatically and steadily in the second half of the 20th century, mainly in industrialized countries of North America and Europe (3). Recent epidemiological studies have shown that the incidence of the disease has begun to stabilize in most industrialized countries and has even decreased in some regions. However, after several decades of increasing incidence, the prevalence of inflammatory bowel disease has risen to more than 0.3% in North America, Australia, and many

countries in Europe (4). Prevalence rates for UC are 249 per 100,000 persons in North America and 505 per 100,000 persons in Europe, whereas incidence rates are 19.2 per 100,000 person-years in North America, 24.3 per 100,000 person-years in Europe, and 6.3 per 100,000 person-years in Asia and the Middle East (3). In newly industrialized countries of Latin America, Eastern Europe, Asia, and Africa, the incidence of inflammatory bowel disease has been increasing rapidly since the 1990s, reflecting the westernization of these societies (4). The peak incidence of the disease occurs between the ages of 15 and 50 years.

1.1.2. Etiology and pathogenesis

While the cause of UC remains uncertain, there is increasing evidence that the disease results from a dysregulated intestinal immune response driven by a complex interplay between the host genotype and intraluminal microbiota (5).

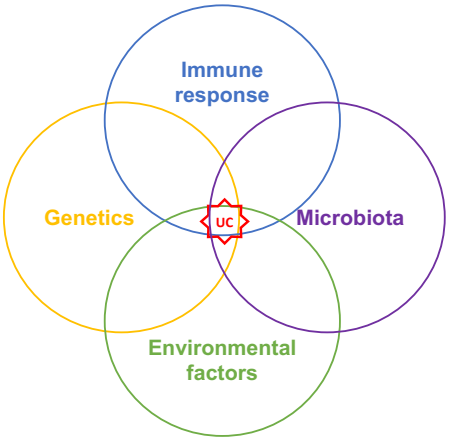

Figure 1. The pathogenesis of Ulcerative colitis requires the interaction of host genetics, immune dysregulation, the gut microbiome, and the external environment.

1.1.2.1. Genetics

Different studies have shed considerable light on the role of genetics in the pathogenesis of IBD. In a large international collaborative study, including over 75,000 patients with Crohn's disease, UC, and healthy controls, 163 distinct genetic risk loci were identified that met genome-wide significance thresholds (6). A substantial portion of these loci

(110/163) confer risk of both diseases, suggesting common disease pathogenesis pathways. Nearly half (50 loci) had identical effect sizes on Crohn's disease and UC, while the remaining were heterogeneous in their effect. Among the remaining 53 unique loci, 23 were specific to UC at the genome-wide significance threshold, though when a lower threshold for significance is used, several of these loci appear to be shared. Several of these loci can be grouped into essential pathways, including the innate immune response, microbial defense and antimicrobial activity, goblet cell function, epithelial restitution, generation of reactive oxygen species, pathways that determine tolerance and training of innate immune cells, and maintenance of balance between Th17 helper T cells and T-regulatory cells (Treg) (7). A recent study has further identified 38 previously unknown disease-associated loci by including individuals of European descent and non-European descent, raising the number of known IBD risk loci to more than 200 to date (8). Together, these loci explain 13.1% and 8.2% of risk in Crohn's disease and UC. However, the vast majority of carriers of these genetic susceptibility polymorphisms will never develop the disease. A familial incidence of UC has been recognized for many years, and although figures vary widely, about 10% to 20% of patients have at least one other affected family member (9). Twin studies only show a concordance in monozygotic twins of around 30% for CD and 15% for UC (10).

1.1.2.2. Immunity

Much of the investigation on IBD pathogenesis has focused on the pathologic immune response. The study of the altered immune response in IBD patients initially concentrated on adaptive immunity, but recent research has highlighted the critical role of the innate immune response in the pathogenesis of IBD (11). (Figure 2). The innate immune response offers the first line of defense against any aggression. It is mediated by various cell types, including immune cells such as neutrophils, monocytes, antigen-presenting cells (APCs) such as macrophages and dendritic cells, and nonimmune cells, like epithelial, endothelial, and mesenchymal cells. Macrophages have been classified as classically activated macrophages (M1) or alternatively activated macrophages (M2) based on their secretory cytokine patterns and pro-inflammatory versus immuno-

regulatory activity (12). Dendritic cells (DCs) are heterogeneous and include myeloid, plasmacytoid, tissue-resident, and blood-monocyte-derived types (13). Their primary function is to monitor the surrounding microenvironment, sample antigens, and set up subsequent immune events, either inducing tolerance or inciting pro-inflammatory responses (14).

Early gut microbial colonization is essential to the development and maturation of the immune system, intending to establish a symbiotic relationship of tolerance and protective immunity (15). Cells involved in innate immune responses sense gut microbiota by recognizing microbe-associated molecular patterns (MAMPs, including pathogen-associated molecular patterns, PAMPs, and damages-associated molecular patterns, DAMPS). Dendritic cells and macrophages activated by microbial compounds, phagocyte luminal antigens and present their peptides associated to HLA class II molecules to activate adaptive immune cells (16). Dendritic cells in the lamina propria actively sample the luminal contents and play a particularly vital role as crucial APC's capable of shaping the immune response. It is now well established that patients with IBD have a defective innate immune response to luminal antigens and several of the genetic variants associated with Crohn's disease lead to a dysregulated innate immune response. NOD2 and the autophagy-related variant ATG16L1 are two variants that are, in particular, related to defects in the innate immune response (17).

Research in the adaptive immune system in IBD has focused on CD4+ T-helper cells (Th cells). There are three types of Th cells involved in the pathogenesis of IBD; Th1, Th2, and Th17. Th1 and Th17 cells play a role in the development of Crohn's disease. Th1 pathway is stimulated by IL-12 generated in response to exposure to infectious agents. The intestinal mucosa of patients with Crohn's disease presents increased concentrations of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), which are critical pro-inflammatory cytokines produced in response to the activation of Th1 cells. Th17 cells secrete IL-17A, IL-17F, IL-21, and IL-22 and are activated by binding IL-23 to surface IL-23 receptors. The secretion of IL-21, in turn, upregulates the expression of the IL-23R, potentiating the inflammation. Mucosal biopsies from IBD patients demonstrate a higher

number of Th17 cells and over-expression of IL-17A (18). An additional type of Th cell subtype that secretes IL-17A and IFN- γ (Th1/Th17) has been described, which may also be involved in IBD's pathogenesis. UC has historically been thought to depend on Th2 cell-mediated responses (19). A specialized type of T cell, the natural killer T cell, seems to mediate the Th2 response in UC (20). However, UC does not appear to be a purely Th2-mediated inflammatory disease as previously believed. Recent progress in the immune mechanisms implicated in UC has recognized that Th17 cells could be a crucial mediator of the tissue damage causing mucosal ulceration of the colon in UC (21). Interleukin-6 (IL-6) is one of the potent inducers of Th17 cells. A study of colonic biopsies from inflamed and noninflamed areas of UC patients showed that IL-6 was the predominant cytokine found in inflamed areas from UC patients, and its concentration correlated with the disease's severity (22). Furthermore, the anti-TNF-α monoclonal antibodies' clinical efficacy in UC supports the crucial pathogenic role of TNF-α in UC (23). The role played by Janus kinase in sustaining Th1/Th17 differentiation has been translated into the therapeutic use of the Janus kinase inhibitor tofacitinib, which has induced a good clinical response in UC patients (24). Moreover, levels of IL-23 (mainly produced by dendritic cells, macrophages, and monocytes) are induced in both CD and UC patients, and this cytokine has been suggested to drive multiple pro-inflammatory pathways in the mucosa (25). Immune cell recruitment into the gut is crucial in maintaining chronic intestinal inflammation through the adhesion of leukocytes to endothelial cells through interaction between adhesion molecules/integrins and chemokines/chemokine receptors. In particular, mucosal addressin cell adhesion molecule-1, which is the specific ligand for α4β7-integrin expressed on the surface of lymphocytes, is over-expressed in the colonic mucosa of patients with UC (26).

Intestinal epithelial cells serve barrier functions and play a role in enteric immunity. This barrier function is achieved partly through intercellular junctions and tight junctions, the intestinal epithelial cells' immune regulatory functions, secretion of antimicrobial peptides, and mucus production by goblet cells. IBD patients demonstrate an alteration in intestinal permeability. Patients with UC have an increased turnover rate of colonic epithelium and other abnormalities of epithelial cells, including reduced metabolism of short-chain fatty

acids, especially butyrate, abnormal membrane permeability, and altered glycoprotein composition mucus produced by the colonic epithelium. Specifically, the mucus layer in UC appears to be thinner than usual (27). Several of the IBD risk loci associated genes, including CDH1, HNF4A, GNA12, MUC19, and ITLN1, play an essential role in maintaining the intestinal epithelial barrier (28). Polymorphisms at the CDH1 locus lead to a truncated form of E-cadherin, resulting in defective localization to the plasma membrane and consequent cytosolic accumulation. The cytosolic accumulation of truncated E-cadherin is associated with goblet cells and Paneth cells' defective maturation and reduced clearance of pathogenic bacteria from the intestinal lumen (29). The deletion of HNF4A causes spontaneous colitis in mice and increases susceptibility to colitis. Intestinal biopsies from patients with UC demonstrate reduced expression of HNF4A (30). PTPN2, a member of the protein tyrosine phosphatase family, and GNA12 encoding the G protein Gα12 maintain tight junction integrity; PTPN2- deficient mice demonstrate greater susceptibility to colitis (31). These and other abnormalities can lead to increased numbers of adherent bacteria, in both the mucus layer and even at the epithelial surface, in patients with UC (32).

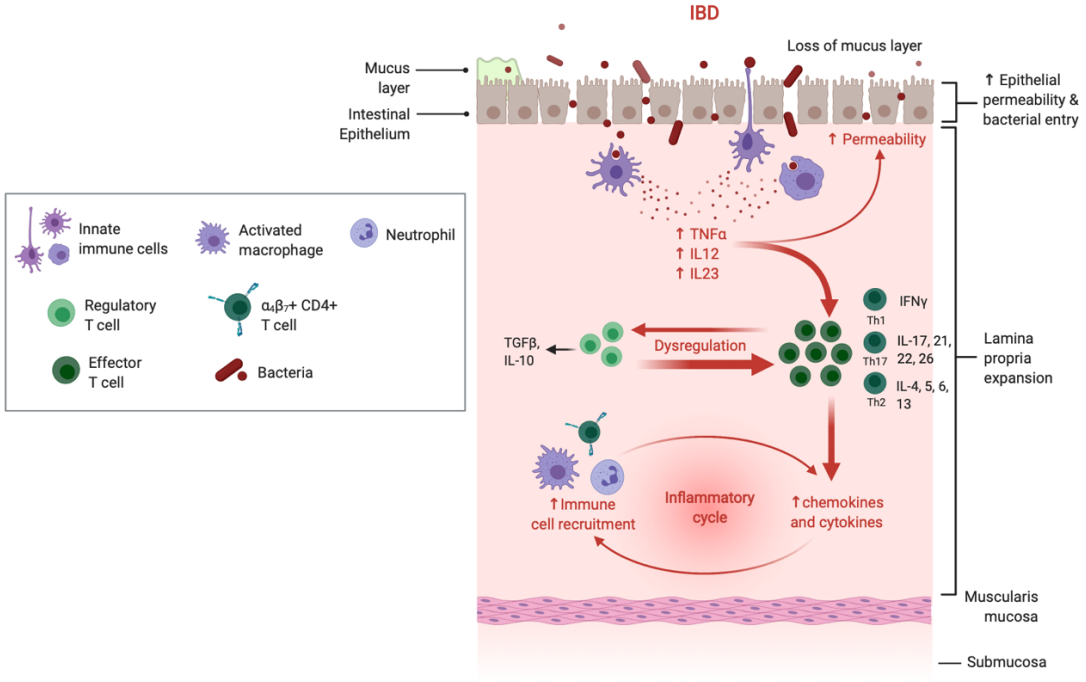

Figure 2. In the presence of pathogenic bacteria, MAMPs stimulate the production of proinflammatory cytokines (IL-1, IL-6 and IL-18 from epithelial cells and IL-6, IL-12 and IL-23 from DCs and macrophages) that induce development of the effector CD4+ T cells TH1 and TH17. TH2 cells are generated that mediate TH2-type responses. Created with BioRender.com

1.1.2.3. Environmental factors

Several cohorts have demonstrated a significant increase in IBD incidence over the past five decades, a trend that cannot be explained solely by genetics. Furthermore, immigrants from low- risk regions migrating to high-risk regions often assume the risk of disease associated with residence. The emergence of IBD in areas where they were previously considered rare, paralleling the "westernization" of lifestyle, also supports an essential role for the environment in disease pathogenesis.

Smoking is one of the most consistently described environmental factor impacting IBD (33). While current smokers have a 2-fold increase in risk for CD, former smoking, within one year of quitting, is associated with a substantial increase in the risk of UC, while current smoking appears to be protective (34). The active ingredients in cigarette smoke that decrease the risk UC have not been defined, nor have the mechanisms of action. Furthermore, current smoking is associated with a more aggressive disease course in CD, greater need for therapy escalation, higher surgery rates, and recurrence following surgical resection. In contrast, ongoing smoking appears to be associated with a milder course and reduced surgery rates in UC. On the assumption that nicotine is the active ingredient in cigarette smoke, transdermal nicotine and nicotine gum trials for the treatment of UC have been performed; in two trials, transdermal nicotine was somewhat effective. Other evidence suggests that carbon monoxide in the inhaled smoke may be mechanistically important. One of the most interesting aspects analyzed to have a possible influence on UC clinical outcomes is appendectomy. A recent study showed that appendectomy might be a protective factor against colectomy, but only if performed before UC diagnosis and in patients operated for appendicitis before 20 years of age (35). In the same study, appendectomy, after UC diagnosis, for acute appendicitis was associated with an increased risk of colectomy, whereas appendectomy without inflammation was not. A meta-analysis, including eight studies, showed that appendectomy does not influence UC patients' colectomy rates.

IBD's pathogenesis is likely the result of continuous antigenic stimulation by commensal enteric bacteria, fungi, or viruses, which leads to chronic inflammation in genetically susceptible hosts who have defects in mucosal barrier function, microbial killing, or immunoregulation. Several infectious organisms, including mycobacteria and viruses, have been studied in the pathogenesis of IBD. However, no specific infective organism has been isolated consistently from patients with UC, and therefore it is unlikely that a common infectious agent causes the disease. It is plausible that disruption of intestinal microbiome composition with antibiotic use, particularly early on in life, may modify disease risk. Indeed, in a nested case-control study of 36 pediatric-onset IBD patients, antibiotic use within the first year of life was more common in those with IBD (58%) than controls (39%) (36). Recent antibiotic use within 2–5 years of diagnosis was also associated with increased risk of adult-onset IBD (37), with the increasing effect seen with a more significant number of antibiotic courses and exposure earlier in life (38). A recent large population-based case-control study found that antibiotic use was an independent risk factor for the development of both CD and UC (39). In contrast, previous studies mostly found an increase in the risk of CD, but not UC.

1.1.3. Diagnosis

1.1.3.1. Clinical manifestations

Patients with UC can present with a variety of symptoms. Common symptoms include diarrhea, rectal bleeding, mucus passage, tenesmus, urgency, and abdominal pain. The onset of symptoms is usually gradual, and symptoms are progressive over several weeks. The symptom complex tends to differ according to the extent of the disease. The severity of symptoms may range from mild disease with four or fewer stools per day with or without blood to more than ten stools per day with abdominal pain and continuous bleeding in severe disease. Fever and weight loss may be prominent in severe or fulminant colitis (40). Around a quarter of patients present with severe acute UC at the onset of their disease, and 15–25% of UC patients develop an acute severe flare necessitating hospitalization during their disease course (41). Patients at risk for aggressive disease

behavior typically are young, nonsmokers, have no previous appendectomy, and present with an extensive disease with deep ulcerations on colonoscopy and high levels of inflammatory biomarkers (42). There are several instruments for determining clinical disease activity in UC. The Simple Clinical Colitis Activity Index (SCCAI) is a questionnaire used to assess UC patients' severity of symptoms (43). Created in 1998, it consists of scores for five clinical criteria, with a score that ranges between 0–19. It is based on clinical symptoms alone. (Table 1). A score of <2.5 points has been shown to correlate with patient-defined remission (44).Composite indices have also been developed, such as the Mayo Clinic score, which includes clinical symptoms, endoscopy, and physician's global assessment (45).

Table 1. Simple Clinical Colitis Activity Index

Symptom	Score
Bowel frequency (per day)	
1-3	0
4-6	1
7-9	2
>9	3
Bowel frequency (per night)	
0	0
1-3	1
4-6	2
Urgency	
None	0
Hurry	1
Immediately	2
Incontinence	3
Blood in stool	
None	0
Trace	1
Occasionally frank	2
Usually frank	3
General well-being	
Very well	0
Slightly below par	1
Poor	2
Very poor	3
Terrible	4
Extracolonic manifestations*	1 x manifestation

**Uveitis, pyoderma gangrenosum, erythema nodosum, arthropathy*

1.1.3.2. Laboratory investigations

Laboratory findings in UC are nonspecific, and patients often have normal laboratory test results if the disease is mild and of limited extent. In contrast, patients with active extensive disease or severe distal disease can demonstrate laboratory abnormalities such as anemia, leukocytosis, and thrombocytosis. Serum inflammatory markers, including erythrocyte sedimentation rate and C-reactive protein (CRP), may be elevated in active disease, while albumin can be decreased in severe cases. A useful inflammatory marker for UC disease activity is fecal calprotectin. Calprotectin is a protein secreted by neutrophils that can be detected in the feces and is, therefore, a marker of intestinal inflammation. Fecal calprotectin can correlate with endoscopic activity. Multiple studies have shown that fecal calprotectin is predictive of relapse in UC; values above 250 μg/g are often predictive of relapse (46), whereas levels below 150 μg/g are often predictive of clinical and endoscopic remission and values ranging from 40.5 to 200 μg/g might also correlate with histological remission (47, 48). However, establishing a calprotectin cut-off is not as straightforward, with different studies that show sensitivity and specificity values varying within the same range.

There is no single test that allows the diagnosis of UC with acceptable sensitivity and specificity. Thus, diagnosis relies on a combination of compatible clinical features, endoscopic appearance, and histologic findings.

1.1.3.3. Endoscopy

The endoscopic hallmark of UC is symmetric and continuous inflammation that begins in the rectum and extends proximally without interruption for the entire extent of disease. The earliest endoscopic sign of UC is a decrease or loss of the typical vascular pattern, with mucosal erythema and edema. As the disease progresses, the mucosa becomes granular and friable. With more severe inflammation, the mucosa may be covered by exudates associated with mucosal ulcerations. In UC, mucosal ulcerations occur in areas of inflammation; they vary in size from a few millimeters to several centimeters and may

be punctate, annular, linear, or serpiginous. Before treatment, these changes are continuous up to a distinct margin of normal-appearing mucosa, or they may extend diffusely to the cecum and, occasionally, into the distal ileum, referred to as backwash ileitis. Limited inflammatory changes in the cecum, especially around the appendix (periappendiceal or cecal red-patch), may be present in patients with distal disease (49, 50). Severe UC is associated with mucosa that bleeds spontaneously, and, with diffuse colitis, there may be extensive areas of denuded mucosa from severe mucosal ulcerations.

Truelove and Witts were the first to report on the sigmoidoscopic appearance of the gut mucosa during a placebo-controlled trial of cortisone for the treatment of active disease (42). Endoscopic lesions were classified as normal or near-normal, improved, or no change or worse. After that, several endoscopic scoring systems were developed using different items and definitions of remission and healing. One of the most frequently used is the Mayo Clinic endoscopic score, part of the Mayo Index. (Figure 3) Mucosal healing can be considered with a Mayo endoscopic score equal to 0 or ≤1 (45).

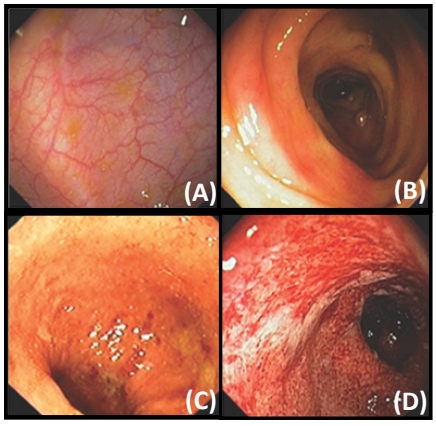

Figure 3. Mayo Endoscopic score. (Photograph from de Lange, T. et al., BMC Gastroenterol. 2004;4:9) (51) (A) Score 0: Normal or inactive, (B) Score 1: Mild (erythema, decreased vascular pattern, mild friability, (C) Score 2: Moderate: Marked erythema, absent vascular pattern, friability, erosions. (D) Score 3: Spontaneous bleeding, ulceration

UC can be classified by the maximal macroscopic extent at colonoscopy (52).

The three subgroups of UC are:

1. Ulcerative proctitis (E1): involvement limited to the rectum (proximal extent of inflammation is distal to the rectosigmoid junction).
2. Left-sided UC (E2): involvement limited to the portion of the colorectum distal to the splenic flexure.
3. Extensive UC (E3) (includes pancolitis): involvement extends proximally to the splenic flexure

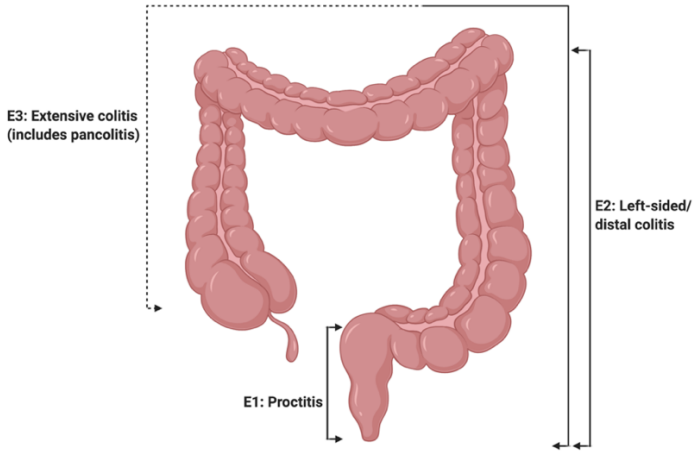

Figure 4. Distribution of Ulcerative colitis. Created with BioRender.com

At the time of initial presentation, approximately 45% of patients with UC have disease limited to the rectosigmoid, 35% have disease extending beyond the sigmoid but not involving the entire colon, and 20% of patients have pancolitis (53). Disease extent influences treatment, whether oral or topical therapy, and determines the onset and frequency of cancer surveillance (54, 55). Proximal disease extension is reported in about 25% of cases. In a recent meta-analysis of 30 epidemiological studies, the overall pooled frequency of UC extension was 22.8%, with colonic extension reported at 17.8% at five years and 31% at ten years (56).

1.1.3.4. Histology

UC is a chronic process with histological features of distorted architecture, and inflammatory infiltrate limited to the mucosa (57-59). Distorted crypt architecture is seen in 57–100%, with crypt branching and atrophy and an irregular villous architecture in 17–

30% (59-61). The inflammatory infiltrate is composed of lymphocytes, plasma cells, and neutrophils, causing cryptitis, defined as the presence of neutrophils within crypt epithelium, and crypt abscesses, defined as the presence of neutrophils within crypt lumen (57, 59). Mucin depletion is not specific for UC. Basal plasmacytosis is the earliest diagnostic feature with the highest predictive value for the diagnosis of UC. It can be observed in 38% of the patients within two weeks after the initial presentation (61). Long-standing disease is characterized by widespread crypt architectural distortion, and the presence of a diffuse increased transmucosal inflammatory cell infiltrate.

Histologically, mucosal healing is characterized by resolution of the crypt architectural distortion and the inflammatory infiltrate. However, the mucosa will still show some sustained damage features, such as a decreased crypt density with branching and shortening of the crypts (62, 63). As for endoscopy, there are many indices to assess histological disease activity in UC since the 1950s (64, 65). The most widely used are the Riley Index (66) and the Geboes Index (67). Although there is to date no agreed preferable scoring system, the Geboes Index is the best validated (kappa for interobserver variation 0.59–0.70) (68).

1.1.4. Medical treatment

Medical therapeutic decisions in UC patients are based upon several important factors, mainly clinical and endoscopic severity of the colitis, the extent of the disease, and patient-related factors (69). Current therapies can be classified into those that treat active disease (induction therapy) and those that prevent disease recurrence once remission is achieved (maintenance therapy). The extent of disease is an important consideration that helps determine the route of administration of medication. (Table 2). Other important factors to consider are a patient's initial response to or side effects from a specific treatment and medication compliance. These factors might favor or preclude the use of a specific agent. The mainstay of medical therapy focuses on regimens that alter host response to decrease mucosal inflammation.

Table 2. Therapies for Ulcerative Colitis Based on Disease Activity and Extent

Activity	Extent		
	Proctitis	**Left-sided colitis**	**Extensive colitis**
Mild	Mesalazine suppository	Topical and oral mesalazine	Topical and oral mesalazine
Moderate	Mesalazine suppository + oral	Topical and oral mesalazine	Topical and oral mesalazine
Severe	Mesalazine suppository + oral therapy; oral/topical steroids Consider admission for IV steroids	Mesalazine + steroids Consider need for admission for IV steroids	Mesalazine + steroids Consider need for admission for IV steroids
Severe - Steroid refractory	Cyclosporine Anti-TNF		
Maintenance	Mesalazine suppositories		
	Oral mesalazine Thiopurines Biologics (infliximab, adalimumab, golimumab, vedolizumab, ustekinumab) Tofacitinib		

Treatments aimed at manipulation of the intestinal microbiota are also being developed for UC. Fecal microbiota transplant (FMT) has been explored in UC. A meta-analysis from 24 UC cohort studies found that FMT induced remission in 33% (70). Three randomized controlled trials also presented promising results regarding the use of FMT to treat UC. From a total of 70 UC patients with active disease, 36 were treated with FMT, and 34 with placebo, once a week for six weeks, and remission was induced in 24% of those treated with FMT compared to 5% in the placebo group (71). Similar results were observed using enemas five days per week for eight weeks, in a study that observed a remission rate of 27% in UC patients treated with FMT when compared to 8% in patients treated with placebo (72). In the third controlled trial, however, the remission rates observed in UC patients treated with FMT from healthy donors were similar to those observed in UC patients receiving their own fecal microbiota (73). In these studies, both placebo and FMT groups were under concomitant anti-inflammatory/immunosuppressive therapy.

1.1.5. Therapeutic goals in ulcerative colitis

Therapeutic goals have changed from treating symptoms towards mucosal healing to modify the natural history of the disease and preserve gut functionality. The concept of deep remission in UC, which includes symptomatic and endoscopic remission, came into view a decade ago when different studies showed the importance of looking beyond symptoms and achieving and maintaining endoscopic healing (74). The treatment goal evolution in IBD has paralleled other immune-mediated diseases, including skin disorders such as psoriasis, in which clinical ambition has peaked with the new concept of disease clearance (75). Endoscopic healing, which is defined as the absence of inflammatory lesions in the colon at endoscopy, is associated with improved short and long-term outcomes (76). Histological remission is being recognized as a significant therapeutic goal and endpoint for UC as histological alterations are often found in the presence of macroscopically normal mucosa seen at endoscopy (77). Evidence indicates that histological remission is a target different from endoscopic healing in UC. It is associated with a lower risk of hospitalization, colectomy, and colorectal cancer.

1.2. Microbiota

Microbial communities are defined as multi-species collections of microorganisms that live and interact with each other in a contiguous environment (78). In humans, the gastrointestinal (GI) tract harbors the vast majority of these microorganisms (79). It has been estimated that the number of microorganisms inhabiting the GI tract exceeds 10^{14}, which would account for more than tenfold the number of human cells and over 100 times the amount of microbiome genomic content as the human genome (80-82). However, a recently revised estimate suggests that the ratio of bacteria to human cells is closer to 1:1 (83). Due to the vast number of bacterial cells in the body, the host and microorganisms inhabiting it are often referred to as a ‘superorganism’ (84). The gut microbial community, also known as gut microbiota, encompasses the collection of eukaryotes (mainly fungi), archaea, bacteria, and viruses. It has co-evolved with the host over thousands of years to form an intricate and mutually beneficial relationship (80, 85).

1.2.1. Techniques to characterize the gut microbiota

One of the significant difficulties in studying the human gut microbiota has been the labor-intensive culture-based methods previously utilized to characterize it. Up to 80% of the microbes observed by microscopic examination of fecal specimens were not recoverable by culture (86). Quantifying the uncultured was severely limited until the development of DNA-based culture-independent methods in the 1980s (87). Culture-independent techniques, which analyze the DNA extracted directly from a sample rather than from individually cultured microbes, allow us to investigate several aspects of microbial communities such as taxonomic diversity and functional metagenomics. (Table 3)

Table 3. Techniques used to characterize the gut microbiota (adapted from (88))

Technique	Description
Culture	Isolation of bacteria on selective media
qPCR*	Amplification and quantification of 16S rRNA. Reaction mixture contains a compound that fluoresces when it binds to double-stranded DNA
DGGE/TGGE#	Gel separation of 16S rRNA amplicons using denaturant or temperature gradient
FISH+	Fluorescently labelled oligonucleotide probes hybridize complementary target 16S rRNA sequences. When hybridization occurs, fluorescence can be enumerated using flow cytometry
Direct sequencing of 16S rRNA amplicons	Massive parallel sequencing of partial 16S rRNA amplicons
Microbiome shotgun sequencing	Massive parallel sequencing of the whole genome

*qPCR, quantitative PCR; #DGGE, denaturing gradient gel electrophoresis/TGGE, temperature gradient gel electrophoresis; +FISH, fluorescence in situ hybridization

1.2.1.1. Culture techniques

Culture was the gold standard for identifying bacterial species for many years, but it is labor-intensive and provides a limited view of the gut microbiota's diversity (89). Furthermore, many gut bacteria are obligate anaerobes, making it more challenging to apply culture-based techniques. Uncultured organisms in the gut microbiota are not necessarily unculturable. They might be culturable, but these organisms' permissive

growth conditions have not yet been consistently developed or even discovered (90). In recent years there has been a renewed interest in microbial culture with the use of culturomics that applies high-throughput culture conditions to study the human microbiota (91).

1.2.1.2. Culture-independent techniques

Next-generation sequencing techniques are currently the most common methods for microbiome characterization. These techniques are either targeted-sequencing or shotgun sequencing on microbial DNA or RNA. Targeted-sequencing methods involve the amplification of marker genes by polymerase chain reaction (PCR) to taxonomically describe a microbial community. One of the most widespread techniques is amplicon sequencing. A single genomic locus is targeted for PCR amplification; the chosen locus or marker must be largely conserved throughout microorganisms of interest but contain sufficient variation to allow the distinction of individual strains or species. Resulting PCR products are sequenced and compared with known reference sequences in a database.

By far, the most common marker is the small or 16S ribosomal RNA subunit gene (92). This 1.5 Kbp gene is referred to as the 16S rRNA. It satisfies a marker's criteria by containing both highly conserved sequences and variable regions that yield a phylogenetic signal, making it a good target for phylogenetic identification (93, 94). 16S rDNA gene contains nine hyper-variable regions (V1-V9) that differ between species. Conserved regions allow the use of universal primers to detect all bacteria present in a sample, whereas hypervariable regions determine which bacteria are being detected (92).

The V4 region is the most prominent V region for achieving reasonable domain specificity, higher coverage, and a broader spectrum of the microbial composition in a sample (95). Sequencing with universal primers results in millions of sequences that correspond to bacteria and archaea in microbial communities. All these 16S rDNA sequences are then stored in several databases such as GreenGenes (96), the Ribosomal Database Project (97), and Silva (98).

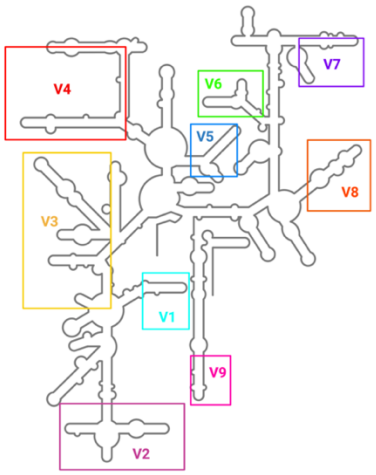

Figure 5. 16S rDNA gene structure. Hypervariable regions differ between bacterial species and allow the classification of microbiota in different phylogenetic categories. Created with BioRender.com

Techniques that target the 16S rRNA gene can demonstrate microbial diversity of the gut microbiota, qualitative and quantitative information on bacterial species, and changes in the gut microbiota in relation to disease. Some degree of sequence divergence is typically allowed. 95%, 97%, or 99% are sequence similarity cutoffs often used in practice, and the resulting cluster of nearly-identical tags is referred to as an Operational Taxonomic Unit (OTU) (99). The assignment of sequences to OTUs is referred to as binning, and it can be performed by clustering of similar sequences and phylogenetic models incorporating mutation rates and evolutionary relationships (100).

Sequencing techniques have evolved in the last years with important advances in sequencing platforms. Most microbiome researchers use the Illumina MiSeq or HiSeq technology that provides millions of raw reads after quality filtering (101). Several tools for analyzing 16S rRNA gene sequences have been developed, such as QIIME (Quantitative Insights Into Microbial Ecology) (102), Mothur (100), UPARSE (103), DADA2 (104), MetaAmp (105), and ANCHOR (106). Most of these tools are based on the clustering of raw sequences into OTUs following different clustering algorithms. They classify each OTU as a bacterial species and generate abundance tables used to compute bacterial diversity and differential abundances. An important concept when dealing with OTU's is that of diversity. Diversity is one of the most widely studied ecologic properties. It is a measure of the taxonomic distribution within a community, either in terms

of distinct taxa or their evolutionary/phylogenetic distance (107). Diversity is widely reported and often promoted as an indicator of health due to its relationships with productivity, functioning, and stability. There are several estimators for calculating diversity. An alpha diversity measure acts as a summary statistic of a single population. A beta diversity measure acts as a similarity score between populations, allowing analysis by sample clustering or dimensionality reductions.

Alpha diversity measures within-sample taxonomic diversity, and it can include the number (richness) and distribution (evenness) of taxa expected within a single population. Various estimators exist to calculate alpha diversity such as the Chao1 (108), Shannon (109), Abundance-based Coverage Estimator (110), and Jackknife (111). On the other hand, Beta diversity measures between-sample taxonomic diversity, describing how many taxa are shared between samples. It can be measured by simple taxa overlap, quantified by the Bray-Curtis index (112) or the UniFrac distance metric (113). Unweighted UniFrac takes into consideration the microbial composition and is insensitive to changes in abundance that may be important to understanding community responses to environmental variation. A later version of UniFrac, called weighted UniFrac, accounts for the microbial composition and the microbial abundance. The standard metric UniFrac determines the fraction of unique branch lengths within a phylogenetic tree (comprising sequences from multiple samples) that is attributable to a particular sample and can be used as a single-sample or multiple-sample similarity measure (114). The more phylogenetically similar communities are, the lower the Unifrac distances, while communities that differ more have higher UniFrac distances. (Figure 6) UniFrac distances can be represented in a distance matrix summarized and visualized in three-dimensions by using principal coordinate analysis (PCoA). PCoA is a dimensionality reduction technique that synthesizes the distances between samples in a scatter plot, where the points (representing the samples) that are more distant from each other are more different (115).

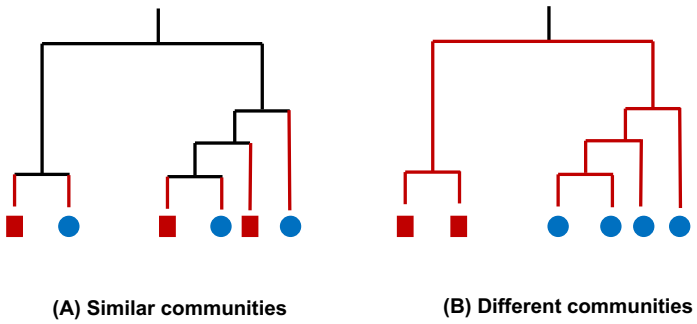

Figure 6. Unweighted UniFrac. UniFrac measures the amount of evolutionary divergence between two communities by dividing the red branches' length by the total branch length of the tree. (A) shows two communities with minimal evolutionary divergence and a relatively low UniFrac distance, (B) shows two communities that are different; their lowest common ancestor is the root of the tree. Adapted from mothur.org

Targeted approaches are also used not only for identifying but also for quantifying microbial DNA. Techniques such as real-time quantitative PCR (qPCR) are based on a targeted marker gene's amplification. Depending on the qPCR primers, this technique allows the detection and quantification of total bacteria, using universal primers, or of a specific group of species if primers specific to a particular group are utilized (116-118). The advantages of qPCR include phylogenetic discrimination, speed, and the inherent ability to design primers to target specific species; it is the most accurate culture-independent measure of the total microbial load. Furthermore, qPCR kits are widely available commercially and can be used by laboratories that do not have access to the other techniques described. Disadvantages include the possibility of PCR bias unless extensive prior analysis has shown it not to occur for a given target–primer combination, and an inability to identify novel species. This technique can also be labor-intensive and technically challenging, for example, primer design.

The molecular approach is not limited to 16S rRNA sequencing. With decreasing costs and increasing speed of DNA next-generation sequencing, coupled with advances in computational analyses of large datasets, it is feasible to analyze entire genomes with good coverage. The resulting information describes the collective genetic content of the community from which functional and metabolic networks can be inferred. Importantly, whole-genome sequencing provides information about nonbacterial community members, including viruses, yeasts, and protists. Traditional metagenomic sequencing

lacks resolution in evaluating the mycobiota composition in the human gut due to bacterial community dominance. Deeper metagenomic sequencing and the extension of fungal databases can enhance sequencing output and fungal species identification (119).

Shotgun sequences of the genomic DNA present in a sample rather than focusing on a specific gene is an approach based on the fragmentation of all the DNA extracted and the sequencing of these small fragments that will be later assembled to build bigger genomic fragments or entire bacterial genomes. Shotgun sequencing allows the identification of most entities present in the microbiome and does not focus only on a specific group as targeted-sequencing. Another advantage of shotgun sequencing is that this technique reaches deeper taxonomic levels providing information at species level whereas 16S rDNA sequencing only reaches genera level with high precision. Moreover, shotgun sequencing provides not only taxonomical information but also allows functional identification. The main drawbacks of shotgun sequencing are its elevated cost in money and time for running the sequence analyses, exceeding by far those of 16S rDNA sequencing. Another issue is the gaps that remain in metagenomics, which correspond to unidentified sequences that may be correlated with an identified organism (120). A recently published database has shown a better resolution in describing the gut microbiome and identifying approximately 70% of the microbial sequences in a sample (121).

1.2.2. Gut microbiota composition

Taxonomically, bacteria are classified according to phyla, classes, orders, families, genera, and species. The dominant gut microbial phyla are Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia, with Firmicutes and Bacteroidetes accounting for more than 90% of the microorganisms present in the GI tract (122-124). The Firmicutes phyla comprises more than 200 different genera such as *Lactobacillus, Bacillus, Clostridium, Enterococcus*, and *Ruminococcus*. *Clostridium* genera represent 95% of the Firmicutes phyla. Bacteroidetes predominantly consists of *Bacteroides* and *Prevotella* genera. The *Bifidobacterium* genus mainly represents the Actinobacteria phylum (122). Although the composition of the gut microbiota has shown

high temporal stability, it can be affected by age, environmental factors such as the use of medications, diet, physical activity, and travel (125-128). The highest persistence is typically observed in the phyla Actinobacteria and Bacteroidetes (129), while Firmicutes is more temporally dynamic (130).

Fungi account for approximately 0.1% of the total microorganisms in the gut (122). The phyla Ascomycota and Basidiomycota are the most abundant taxa in healthy individuals' mucosal samples (131). The human gut mycobiome is low in diversity and dominated by yeast, including *Saccharomyces, Malassezia*, and *Candida*. It has been observed that both inter- and intra-subject variability is high, revealing that unlike bacterial communities, an individual's mycobiome is no more similar to itself over time than to another person's, making it less temporal stable (132). Recent insights have suggested that gut mycobiota may be intricately linked to health and disease. Evaluation of the gut mycobiota has shown that not only are the fungal communities altered in disease, but they also play a role in maintaining intestinal homeostasis and influencing systemic immunity (133, 134).

Direct sequencing of fungal DNA has been the primary method used to characterize the mycobiome, mainly using the 18s small subunit and the large 28s subunit of rDNA. However, traditional metagenomic sequencing does not have sufficient resolution to evaluate the gut mycobiome's composition due, in part, to the dominant abundance of the bacterial community (122). Unlike the bacterial 16s rDNA sequences for which large databases are available, sequences available for fungi in the NCBI GenBank are much more incomplete. It is estimated that less than 1% of fungal species are represented. Due to these limitations, the coding technique known as ITS ("internal transcribed spacer") has been used, specifically targeting the mycobiome (132). (Figure 7)

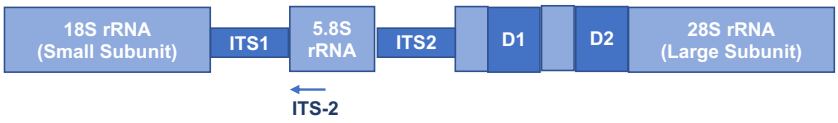

Figure 7. Diagram of the ribosomal gene complex. *Includes a sequence coding for the 18S rRNA gene, an internal transcribed spacer region1 (ITS1), the 5.8S rRNA gene coding region, ITS2 and the sequence coding for the 28S rRNA gene. The arrow indicates the approximate position of primers for PCR based amplification of the region. Adapted from (135)*

The microbiome's viral component (gut virome) is dominated by bacteriophages (136), which are believed to play crucial roles in shaping microbial communities through predation and horizontal gene transfer (137). These complex phage communities represent one of the biggest gaps in our understanding of the human microbiome. Bacteriophages play vital roles in many microbial communities by driving diversity, aiding nutrient turnover, and facilitating horizontal gene transfer. Through their various effects on bacteria, ranging from cell lysis to the transfer of genetic material encoding toxins or resistance to antibiotics, phages can confer differential fitness on their hosts and influence the gut's microbial composition. The predominant viral order is Caudovirales that encompasses most of the known phages (138). The development of new techniques is improving the characterization of gut virome. Archaea also contribute to a small proportion of the gut microbiota. The most predominant Archaea in the gut is *Methanobrevibacter*. Methanogenic archaea reduce hydrogen levels via the production of methane, thereby stimulating food fermentation by saccharolytic bacteria (139).

The gut microbiota can be considered an additional organ that communicates with the host and can develop functions that the host cannot perform by itself. There is ample evidence showing that the microbial colonizers are a constitutive and functional part of the human host. The gut microbiota is involved in health and disease status due to its influence in the host's immune function, nutrition, and protection against pathogens (140, 141). It also plays an essential role in the digestion of specific food substances that otherwise could not be degraded, such as dietary carbohydrates (142). The gut microbiota helps the host absorb and metabolize these nutrients and, in return, it lives and replicates in a nutrient-enriched niche provided by the host. Gut microbes promote body development by prompting transcription of host genes, regulate immunity, provide a metagenome that expands the host's metabolic competence, and influence behavior.

1.2.3. Dysbiosis

High diversity and richness are commonly associated with health status. Dysbiosis has been broadly defined as an imbalance in the composition and metabolic capacity in the gut microbiota (143). Dysbiosis is associated with decreased diversity owing to a shift in

the balance between commensal and potentially pathogenic microorganisms (144). There are multiple ways that the microbial community structure can be influenced; the host's genetics, diet, infection, or medical interventions (such as antibiotics). The hygiene hypothesis originally proposed that antibiotic usage and lifestyle alterations that limit microbial exposure were predisposing populations of people in Western countries to autoimmune disease (145). Antibiotic use reduces bacterial diversity of the gut microbiome, and antibiotic exposure may have long-term consequences (146). Observational studies have shown that frequent use of different antibiotics in early life increases the risk of presenting immuno-inflammatory and metabolic diseases later in life, including obesity, allergies, asthma, celiac disease, and IBD, as well as susceptibility to infections (147). Exaggerated immuno-inflammatory responses against resident microbes characterize IBD. Numerous microbiome studies have found a lower microbial richness and diversity in the gut microbiome of IBD patients than healthy controls (144, 148, 149). The drop is especially manifested during and after flares of disease (150). Rupture of the symbiotic balance due to the mismatch between gut microbes and mucosal immunity results in a loss of diversity, suggesting the selection of oxygen-resistant species (151, 152). Although dysbiosis has long been recognized as a hallmark in IBD (148, 153-156), other diseases such as obesity (157), metabolic syndrome(158), diabetes (159), irritable bowel syndrome (160), asthma (161), allergies and even autism (162) have also been linked with alterations in the gut microbiota. It remains unknown if these alterations in the gut microbiota are the cause or consequence of the disease, but animal models have provided some insight (144). Germ-free mice have impaired immune development and epithelial repair (163, 164). A study with separate groups of germ-free mice colonized with fecal microbiota from human adult twins discordant for obesity showed that mice that received obese fecal microbiota presented more significant increases in body mass and adiposity (165). In mouse models of IBD, dextran sodium sulfate-induced colitis mice showed an elevated abundance of *Enterobacteriaceae*, with a correlation with disease activity (166). T-bet−/−RAG−/− ulcerative colitis (TRUC) mice are models of spontaneous colitis, but germ-free TRUC mice did not develop colitis, thus underscoring the importance of the microbiota on the development of IBD (167). Definitive cause-effect mechanistic relationships are challenging to prove outside of specific animal

models, and well-designed, longitudinal, cohort studies in human subjects will be required to demonstrate causality.

1.3. Ulcerative colitis and gut microbiota

As stated previously, the etiology of UC remains uncertain; it is thought to be the result of an inadequate immune response to microbiota in genetically susceptible individuals. Many IBD susceptibility loci suggest an impaired response to microbes in disease, but the causality of this relationship is unclear. Observations supporting a role for the gut microbiota in IBD include the predisposition of inflammation for anatomical regions with relative fecal stasis (terminal ileum and rectum), the effectiveness of fecal diversion as a treatment for Crohn's disease, and the rapidly increasing incidence of IBD globally associated with industrialization and accompanying alterations in diet and environmental exposures (144). IBD has been associated with dysbiosis, defined as a decrease in gut microbial diversity owing to a shift in the balance between commensal and potentially pathogenic microorganisms or pathobionts (commensal organisms that, under specific environmental or genetic influences, can cause or promote disease) (168). The intestinal microbiota of patients with UC shows decreased diversity, less stability, and overexpression of certain species. Although global changes in the microbiota of IBD patients have been documented, strong evidence for the existence of specific pathobionts that cause IBD is limited.

1.3.1. Bacterial diversity and stability

In mucosa samples from UC patients, there is a reduction in bacterial diversity determined both by 16S rDNA amplification and sequencing. The decline in diversity is characterized by a loss of anaerobic bacterial species such as *Bacteroides, Eubacteria, Lactobacillus*, and other commensal bacteria, mainly of the Phyla Firmicutes and Bacteroidetes (169, 170). Very similar findings have also been found in pediatric UC patients admitted for a severe flare (171). A study in twins discordant for UC analyzed the 16S rDNA sequences in biopsy samples of the left colon mucosa, finding less bacterial diversity in twins with UC than in healthy ones, with more presence of Actinobacteria and Proteobacteria and

less of Bacteroidetes (172). The lower proportion of Bacteroidetes in UC patients is mainly due to a reduction in the Prevotellaceae family. Interestingly, the healthy twins from the discordant couples also showed less diversity than healthy individuals without an affected twin, which would support the idea that there is a certain degree of inheritance in these changes. However, a similar study carried out in 40 pairs of twins, in which at least one of them had IBD, in this case, fecal samples, found no differences between the twin with UC in remission and the healthy twin (173). In another recent study of discordant twins for UC in pediatric age, but with a metagenomic approach, a reduction in global bacterial diversity was evidenced with significant results only for Clostridiales (174).

The reduction in UC patients' species diversity is also associated with a temporary instability of the dominant taxa. In fecal samples collected sequentially during one year of follow-up of patients with UC in stable remission with treatment, only one-third of the dominant taxa were detected persistently, with a low rate of similarity between samples from the same individual at the beginning and end of the tracing. In contrast, healthy controls showed high stability, with similarity rates of around 80% (175).

1.3.2. Specific modifications of the microbiota: aggressive and protective bacteria

In addition to the reduction in diversity and stability, UC patients present specific modifications of the intestinal microbial composition, especially an increase in the presence of aggressive bacterial species. One of the prominent findings in morphological studies in colon biopsy samples from patients with active UC is the increased density of bacteria adhering to the colonic epithelium that are not present in healthy individuals with a normal colonoscopy (176). Furthermore, not only does the concentration of bacteria increase, but bacteria with aggressive characteristics appear.

In samples of inflamed colon biopsies from UC patients, bacterial species capable of invading the epithelium such as *Fusobacterium varium* are identified (177). *F. varium* isolated from the colon mucosa of UC patients and its culture supernatant is cytotoxic to epithelial cells in vitro. *F. varium* adheres to colon epithelial cells invading the cytoplasm and inducing the cells to produce pro-inflammatory cytokines such as IL-8, TNFα, and IL-

6 (178). In another study in which biopsies were collected from patients with active UC, active CD, and ischemic colitis, *F. varium* was found in UC patients' biopsies more frequently than in the other two groups (177). Studies in patients with refractory UC in which antibiotic regimens directed against *F. varium* induce some clinical improvement in the short term (179-182). Some strains of *Fusobacterium nucleatum*, a bacterium related to colon cancer, isolated from biopsies of inflamed mucosa from UC patients, also present invasive characteristics (183). Regarding *Escherichia coli*, some studies show that it is increased in fecal samples and associated with the mucosa in patients with UC, although to a lesser extent than in patients with CD. Some *Escherichia coli* show aggressive characteristics and invasiveness, suggesting that they could alter the intestinal mucosa barrier (184, 185). A study of the mucus layer associated with crypts obtained from biopsies from the colon of UC patients by laser micro-dissection has shown an increase in the number of *Desulfovibrio* subspecies by PCR. These are sulfate-reducing, Gram-negative anaerobic bacteria potentially involved in UC's pathogenesis due to their ability to generate sulfur (186).

Some bacteria are underrepresented, such as *Faecalibacterium prausnitzii*, one of the most representative of the Clostridium leptum group, a butyrate-producing bacterium with anti-inflammatory properties that is decreased in patients with UC both in activity and in remission (187). In a study of 116 patients with UC in remission, a lower amount of *F. prausnitzii* was associated with a shorter time in remission and a higher number of relapses. Likewise, the levels of *F. prausnitziii* recover progressively after an outbreak, and those patients who remained in prolonged remission had higher levels than those who returned to have a flare-up of inflammation (188). A recent study that compared discordant sibling pairs for UC also revealed a significant reduction in *F. prausnitzii* using metagenomics techniques (174). *Coprococcus,* another butyrate-producing member of Clostridia, is also decreased in IBD (156). A case-control study in a newly-diagnosed IBD pediatric population found that *Coprococcus* was decreased in fecal samples of cases compared to controls and further decreased in non-responders compared to responders (189). This finding was also confirmed in a study that explored mucosal and stool samples collected from a treatment-naïve pediatric CD cohort. *Coproccocus* was negatively

associated with CD. (148) A study in UC patients who received fecal microbiota transplant (FMT) from either healthy donor or autologous feces found a decrease in *Coproccocus* in basal (pre-FMT) fecal samples of UC patients when compared to healthy donors (190). Furthermore, the presence of *Coprococcus eutactus* in basal samples of UC patients was a determinant of response to autologous FMT.

A decrease in *Akkermansia muciniphila* has also been found in patients with UC (191). *A. muciniphila* is a bacterium attached to mucus, degrading mucin from where it extracts its primary source of nutrients. *A. muciniphila* has been linked to intestinal health, adheres to the intestinal epithelium, and increases the integrity of the enterocyte layer "in vitro", suggesting that it can strengthen the intestinal barrier (192). A study in UC patients who received healthy donor FMT found that a higher abundance of *A. muciniphila* in the donor microbiota was associated with response to FMT in these patients (193). *Eubacterium rectale*, a bacterium that produces short-chain fatty acids, is decreased in adults and children UC patients, compared to healthy controls and healthy siblings, respectively, and through different microbiota study techniques (194-196).

Treatments used in inflammatory bowel disease can also induce changes in the microbiota. Although it included few patients, one study detected a lower diversity of the fecal microbiota analyzed by pyrosequencing of 16srDNA in patients treated with thiopurines (197).

Some factors such as tobacco also induce important changes that may explain, at least in part, the relationship of tobacco with inflammatory bowel disease. Subjects who smoke, healthy individuals, and IBD patients have higher amounts of *Bacteroides* and *Prevotella* in their feces. When they quit smoking, the most important changes are an increase in Firmicutes and Actinobacteria and a decrease in Bacteroidetes and Proteobacteria (198).

1.3.3. The non-bacterial microbiota and IBD

Most studies investigating the link between inflammation and the microbiota have focused on bacteria. The gut microbiome also includes fungi and viruses, and the role of these microorganisms in health and disease is being increasingly recognized.

1.3.3.1. Gut Mycobiome in IBD

Fungi have long been suspected of playing a role in the pathogenesis of inflammatory bowel disease. The antibody, Anti-*Saccharomyces cerevisiae* antibody (ASCA) has been used to target *Saccharomyces cerevisiae* cell wall antigens to diagnose CD (199). Nevertheless, *Candida albicans* also express common β-glucan epitopes similar to that of *S. cerevisiae*, suggesting that ASCA as a diagnostic tool may not be specific enough to detect individual fungi (200). Furthermore, several genes associated with inflammatory diseases, such as Card9, are involved in the immune response against fungi (201). In healthy subjects, the phylum Ascomycota and Basidiomycota are the most abundant with Saccharomycetes and Tremellomycetes as the dominant classes of Ascomycota and Basidiomycota, respectively. *Saccharomyces, Candida*, and *Cladosporium* are the most abundant genera. The mycobiome appears to be less stable and more susceptible to episodic fluctuations than the bacterial microbiota (202).

Early studies exploring the gut mycobiota in IBD using DGGE have found increased fungal diversity in fecal samples from IBD patients (203, 204). A study in pediatric patients with IBD that sequenced the ITS1 region's gene found a higher abundance of *Candida* species compared to healthy controls (205). Another study in adult subjects determined the composition of the fecal mycobiota in 235 patients with IBD and 38 healthy controls using ITS2 sequencing (156). A decrease in fungal diversity was found only in patients with UC with an increase in the Basidiomycota / Ascomycota ratio in patients with IBD. Despite the substantial reduction in Ascomycota in IBD patients, the abundance of *C. albicans* was found to be increased while *S. cerevisiae* was decreased. Although both species belong to the Ascomycota phylum, *S. cerevisiae* competes with the colonization and adherence of *C. albicans*, preventing its transformation into invasive hypha, which may explain, in part, the results (206).

The mycobiota associated with the intestinal mucosa has also been evaluated in different studies. In a study in 23 patients with CD in remission, active, and healthy controls, mucosa samples obtained during ileocecal resection or colonoscopy were analyzed (131). The number of fungi was determined by qPCR through 18s rRNA amplification in

addition to ITS2 sequencing. This study confirmed that, both in healthy subjects and in CD patients, Basidiomycota and Ascomycota are the dominant phyla. In the mucosa of patients with CD, a greater abundance of *Candida glabrata* species was found in inflamed mucosa and *S. cerevisiae* in non-inflamed mucosa. Another study also explored fungi associated with the intestinal mucosa in CD patients and healthy controls (207). The samples from patients with CD showed a significant decrease in Ascomycota's abundance with an increase in Basidiomycota, similar to the studies previously described. It was observed in CD patients that the genus *Malassezia* was responsible for the rise in Basidiomycetes, especially in those with a pattern of ileocecal involvement. At the same time, *Malassezia* was rarely found in samples from healthy subjects.

Studies have shown the mycobiota interaction with other intestinal microbiota components such as bacteria (trans-kingdom relationships) and its possible association with IBD. A positive correlation in abundance was found between *Candida tropicalis, Serratia marcescens*, and *Escherichia coli* in samples from CD patients compared to healthy subjects. In this same study, an "in vitro" model was performed that further demonstrated the synergy between these three microorganisms by showing a combined effect on the formation of a polymicrobial biofilm. Hypothetically, although the Candida species by themselves are not the only contributor to IBD development, they could play a regulatory role in the relationship of microorganisms from different kingdoms in IBD pathogenesis (208).

1.3.3.2. Gut Virome in IBD

One of the first studies that characterized gut virome dysbiosis as a condition associated with IBD showed an increased abundance of Clostridiales-, Alteromonadales- and *Clostridium acetobutylicum*-infecting phages, as well as a higher abundance of *Retroviridae* in IBD in comparison with healthy individuals (209). A study in UC patients showed that Caudovirales bacteriophage expansion, coupled with a decrease in diversity, richness, and evenness, occurred in UC patients, directly correlating with intestinal inflammation. Escherichia phage and Enterobacteria phage were enriched in UC mucosa when compared to controls (210). In a study that compared subjects with IBD with healthy

controls who lived in the same household showed that IBD patients had a significantly more significant expansion of the taxonomic richness of bacteriophages of the order Caudovirales and that these alterations did not reflect changes in the bacterial community (211). Some of these findings were also observed in another study in ileum biopsies from pediatric CD patients (212). *Faecalibacterium prausnitzii* prophages were more prevalent or more abundant in a study in fecal samples from IBD patients than healthy controls, suggesting that these phages could play a role in the pathophysiology of the disease (213). This finding supports the notion of studying the virome simultaneously with the bacteriome to obtain a holistic picture of the intestinal ecosystem changes in a disease such as IBD. In this regard, a recent study (214) applied the complete virome analysis (WVA) protocol to the data set previously published (211), obtaining new insights into the composition of the virome/bacteriome in IBD. The possible effect of bacteriophages in promoting chronic inflammation was highlighted by a study describing their ability to impact gut immune response in germ-free mice. Herein, *Lactobacillus, Escherichia*, and *Bacteroides* bacteriophages and phage DNA induced IFN-g production via TLR9. Moreover, by increasing bacteriophage levels, colitis was exacerbated via TLR9 and IFN-g (211).

To summarize, there is an imbalance of the intestinal microbiota in patients with IBD, called dysbiosis. However, it has not been possible to establish a direct causal relationship with the disease's development. Studies of the microbiota in inflammatory disease have focused on bacteria, with relatively little knowledge about other microorganisms, including fungi and viruses, and how they interact. Thanks to new technologies for studying the microbiota that are independent of conventional culture-techniques, enormous progress has been made in its knowledge; however, many gaps in knowledge remain to be solved, especially concerning the functional repercussion of the changes observed in IBD.

HYPOTHESIS

The etiology of UC involves an aberrant immune response to an imbalanced gut microbiota in genetically susceptible individuals, following unknown triggering events. Microbiome studies have found a lower microbial richness and diversity in the gut microbiome of IBD patients when compared to healthy controls, and the drop is especially manifested during and after flares of disease. The gut microbiome is essential in maintaining health and mediating illness, and environmental and host factors influence its composition.

Clinical, endoscopic, and histological remission positively impacts on the natural history of UC. Reaching these goals impacts the natural history of UC, probably in the direction of true disease modification.

We hypothesized that UC patients who reach clinical, endoscopic, and histological remission for an extended period would also present a different gut microbiota composition than UC patients with shorter remission lengths or active disease. UC patients in long remission will show a gut microbiota that will be similar to healthy controls.

We believe that a gut microbiota that is more similar to healthy individuals in long-term remission UC patients could potentially be a goal to pursue to define disease remission further.

OBJECTIVES

MAIN OBJECTIVE

Characterize the fecal gut microbiota of UC patients in long-term quiescent disease and compare it to UC patients in short-term remission, UC patients with active disease, and healthy, unrelated subjects.

SECONDARY OBJECTIVES

a) Define specific fecal gut microbiota characteristics and their correlations with clinical outcomes at two years follow-up in UC patients.

b) Determine the association of gut microbiota characteristics with UC patient's clinical data.

c) Explore and compare the fungal and bacterial load in fecal samples from UC patients' groups and healthy controls.

4.1. Study design

A prospective, observational study of adult individuals, 18 years or older was performed.

Four groups of subjects were studied: UC patients in long remission, UC patients in short remission, UC patients at the onset of disease flare, and healthy non-related individuals.

- Long remission was defined as at least five years of flare-free disease with clinical (SCCAI (clinical activity index) <2 (43)), endoscopic (Mayo endoscopic score <1 (45)) and histological (Geobes score <1 (67)) remission at time of inclusion.
- Short remission was defined as clinical remission between 3 and 12 months and fecal calprotectin ≤150 ug/g at inclusion.
- Flare was defined as an active clinical disease with a SCCAI > 4 and fecal calprotectin >150 ug/g.
- Healthy individuals

UC patients with left-sided or extensive UC (either E2 or E3 by the Montreal classification (52)) and healthy controls were included. The diagnosis of UC was established by clinical, endoscopic, and histologic criteria. Recruited healthy controls had no personal or family history of IBD. Subjects were excluded if they were unable/unwilling to provide informed consent, had severe comorbidities, were pregnant, had concomitant primary sclerosing cholangitis, took antibiotics or probiotics within the previous two months, or had isolated rectal UC. All patients were on stable medication at the time of sampling.

Ethical approval for the study was granted by the local Ethical Committee (PR(AG)333/2015). Written informed consent was obtained from all participants.

Demographic, clinical, and biochemical data were collected during clinic visits. These included the type of treatment received, duration of disease, Montreal classification,

gender, tobacco use, CRP, and calprotectin, among others. All patients were asked to follow their regular diet before data and sample collection.
A subset of UC patients (long and short remission groups) was further followed for up to 24 months afterward to determine clinical activity of the disease.

4.2. Sample collection

Two stool samples (eight weeks apart) were obtained from all subjects except from the UC flare group from which only one sample was collected at the onset of a flare, before prescription of changes in medication.
Samples were self-collected at home an immediately frozen by the participants in their home freezer at −20°C and brought no later than one week after collection to the laboratory in a freezer pack, where they were stored at −80°C. Aliquots of around 250mg were obtained on solid CO2 (dry ice) to maintain the sample's frozen status to avoid the degradation of nucleic acids.

4.3. DNA extraction from fecal samples

Before starting the extraction procedure, 800mg of 0.1mm Zirconia/Silica beads were weighed and deposited in UV sterilized tubes. DNA was extracted following the International Human Microbiome Standards (IHMS; http://www. microbiome-standards.org). A frozen aliquot (250 mg) of each sample was suspended in 250μL of guanidine thiocyanate, 40 μL of 10% N-lauroyl sarcosine, and 500 μL of 5% N-lauroyl sarcosine. To ensure the cell wall disruption of gram-positive bacteria and avoid introducing a bias in the recovery of all bacteria, mechanical disruption was performed using a Beadbeater (Biospec Products) (215). Poly Vinyl Poly-Pyrrolidone (PVPP) was added in multiple washing steps to precipitate and discard molecules such as aromatic molecules, nucleic debris, cellular debris, or proteins. To clear lysates, enzymatic digestion of RNA was performed. The resulting DNA from the previous step was

precipitated and ethanol-purified (216). Pure DNA was resuspended in 200µl Tris-EDTA buffer and stored at -20°C until further analysis. (Figure 8).

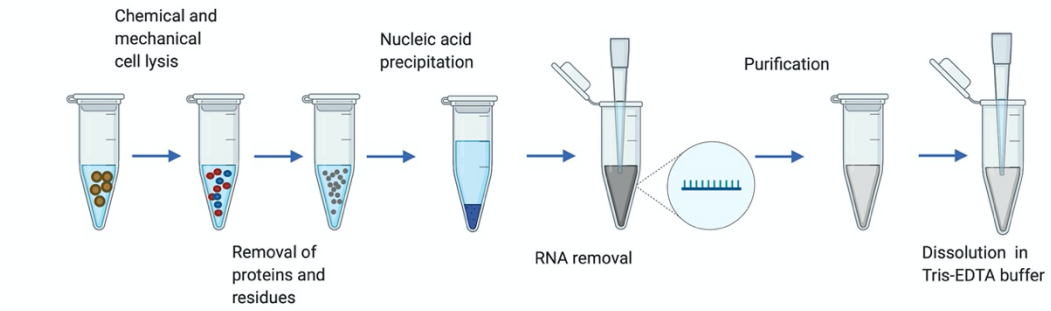

Figure 8. Summary of steps of manual protocol for DNA extraction. Created with BioRender.com

4.4. 16S rRNA gene amplification for sequencing

To profile the microbiome composition, PCR amplification of the hypervariable region (V4) of bacterial and archaeal 16S rRNA gene was performed. The 5' ends of the forward (V4F_515_19: 5' - GTGCCAGCAMGCCGCGGTAA -3') and reverse (V4R_806_20: 5' - GGACTACCAGGGTATCTAAT -3') primers targeting the 16S gene were tagged with specific sequences for Illumina® MiSeq Technology as shown in Table 4. Multiplex identifiers, known as Golay codes, with 12 bases and were specified downstream of the reverse primer sequence (V4R_806_20) (102, 217).

Table 4. Primers used for 16S rRNA gene amplification of Illumina MiSeq sequencing

Primer type	SEQUENCE 5' → 3' ILUMINA FLOWCELL - BARCODE - ADAPTER - LINKER - V4 REGION
Forward	AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
Reverse	CAAGCAGAAGACGGCATACGAGATXXXXXXXXXXXXAGTCAGTCAGCCGGACTACHVGGGTWT CTAAT

Standard PCR using 0.75 units of Taq polymerase (AmpliTaq Gold, Life Technologies®) and 20 pmol/μL of the forward and reverse primers (IDT Technologies®) was run in a Mastercycler gradient (Eppendorf) at 94°C for 3 min, followed by 35 cycles of 94°C for 45s, 56°C for 60s, 72°C for 90s and a final cycle of 72°C for 10 min.

A 1% agarose gel stained with ethidium bromide was performed and ran in a 1x Acetate EDTA (TAE) buffer. Five μl of PCR product was mixed with a 6x loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and placed in wells of solidified agarose gel along with 100 bp DNA Molecular Weight Marker XIV (Roche®) at about 90-100V for 35 to 45 minutes to visualize the amplicon bands in a Gel Doc XR+ system (Bio-Rad®).

PCR amplification was confirmed by the appearance of amplicon bands. The absence of bands could be explained by the presence of too little genomic template DNA in the sample or PCR inhibitors in the sample. We diluted the genomic DNA as an attempt to get rid of inhibitors. After confirmation that the targeted gene was amplified, amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) and quantified using a NanoDrop ND-1000 Spectrophotometer (Nucliber®). DNA integrity was examined by micro-capillary electrophoresis using an Agilent 2100 Bioanalyzer with the DNA 12000 kit, which resolves the distribution of double-stranded DNA fragments up to 17000 bp in length.

The pooled amplicons (240 ng of DNA per sample) were sequenced using Illumina MiSeq technology at the genomics core of the Autonomous University of Barcelona (UAB, Spain), following standard Illumina platform protocols.

4.5. Illumina sequencing

The sequencing process, as described in the Illumina website: “Sequencing templates are immobilized on a proprietary flow cell surface designed to present the DNA in a

manner that facilitates access to enzymes while ensuring high stability of surface-bound template and low non-specific binding of fluorescently labeled nucleotides. Solid-phase amplification creates up to 1,000 identical copies of each single template molecule in close proximity (diameter of one micron or less). Sequencing by synthesis technology uses four fluorescently labeled nucleotides to sequence the tens of millions of clusters on the flow cell surface in parallel. A single labeled deoxyribonucleoside triphosphate (dNTP) is added to the nucleic acid chain during each sequencing cycle. The nucleotide label serves as a terminator for polymerization, so after each dNTP incorporation, the fluorescent dye is imaged to identify the base and then enzymatically cleaved to allow incorporation of the next nucleotide. Since all four reversible terminator-bound dNTPs (A, C, T, G) are present as single, separate molecules, natural competition minimizes incorporation bias. Base calls are made directly from signal intensity measurements during each cycle, which greatly reduces raw error rates" (Figure 9).

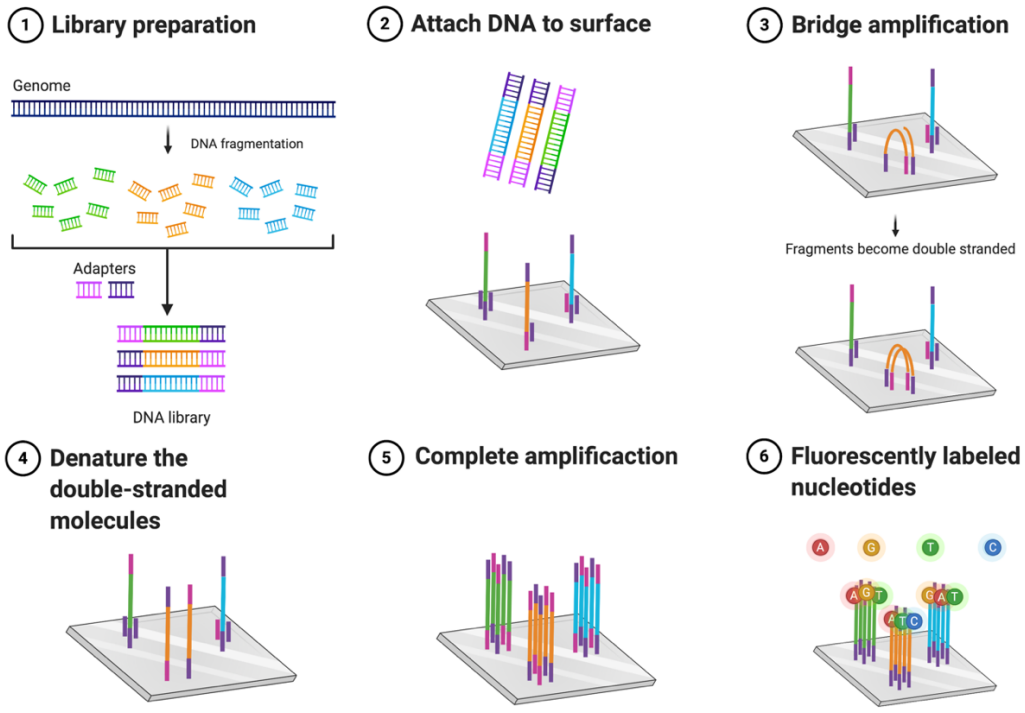

Figure 9. Diagram of Illumina sequencing technology. Adapted from www.illumina.com. Created with BioRender.com

4.6. 16S rRNA sequencing

For 16S rRNA amplicons, pools of equally concentrated samples (240 ng of DNA per sample) were prepared and later diluted to 2 nM. Then, denatured templates were further diluted to 5 ρM and subsequently combined to give an 85% 16S rRNA gene amplicon library and 15% PhiX control pool. Sequencing with Illumina MiSeq technology was performed, as described in the Illumina sequencing section, at the genomics core of the Autonomous University of Barcelona (UAB, Spain). Single-end sequences were received and later analyzed.

4.7. Database preparation for 16S rRNA analyses

We performed 16S rRNA analyses combining the Greengenes (version gg_13_8) and the PATRIC (Pathosystems Resource Integration Center) databases. The Greengenes database contains 16S rRNA gene annotated sequences from Bacteria and Archaea, and the PATRIC database is composed of all known pathogens, many of which are not present in Greengenes.

To combine the Greengenes release gg_13_8 and the PATRIC databases, we extracted the 16S rRNA sequences and their taxonomical annotation from the annotated genomes in PATRIC, avoiding different strains of the same bacterial species. We formatted both sequences and taxonomical annotations from PATRIC to QIIME compatible files. Finally, we combined the obtained PATRIC files with the Greengenes database into a single database. Due to annotation differences in both databases, we found repetitions in some genera. To fix this problem, we changed taxonomical annotation for those repeated genera using PATRIC annotation as it was the most recent one.

4.8. 16S rRNA sequences analyses

The bioinformatics pipeline used for data analysis was Quantitative Insights Into Microbial Ecology (QIIME) [www.qiime.org] (102). Briefly, the process is divided into two stages: upstream and downstream. The former process includes the raw data processing with appropriate file generation for the subsequent microbial analysis carried out during the downstream step. Before the upstream analysis, metadata containing sample identifiers, barcodes, primer sequence, time point, sample status, clinical information of the subjects involved in the studies, and other additional information of the samples needed for the analyses was properly filled out.

Then, the raw sequences were loaded into the QIIME 1.9.1 pipeline. Low-quality sequence reads were filtered out by applying default settings and a minimum acceptable Phred score of 20. Correct primer and barcode sequences were checked. After filtering, from a total of 181 fecal samples, we obtained 8336717 high-quality sequences, with a number of reads ranging from 1108 to 94546 per sample. We used the USEARCH (218) algorithm to cluster similar filtered sequences into Operational Taxonomic Units (OTUs) based on a 97% similarity threshold. Chimeric sequences were identified and removed with UCHIME (219).

A representative sequence was picked from each OTU. Representative sequences were aligned against GreenGenes template (gg_13_8 release). A taxonomical assignment step was performed using the basic local alignment search tool to map each representative sequence against a combined database encompassing Greengenes and PATRIC (Pathosystems Resource Integration Center) databases. We built the OTU table that contains the taxonomical assignment per OTU and their relative abundances in each sample. Relative abundance refers to how common or rare a species is relative to other species in a given sample. Thus, each taxon's relative abundance was expressed as the proportion of the taxon over the total taxa that is 1.0.

To correctly define species richness for the analysis of between-sample diversity, known as beta diversity, the OTU table was rarefied at 1607 sequences per sample and kept for further analysis of a total of 180 samples and 4.064.266 reads. The summarized taxa table was used to classify taxa from the Domain to the Species level.

4.9. Diversity

OTU tables were rarified to perform diversity analyses to normalize and overcome cases in which samples have a different number of sequences. To estimate microbial richness and evenness of the sample, known as alpha diversity, Chao1 and Shannon indexes (220, 221) were calculated. Between-samples diversity or beta diversity was computed using the weighted and unweighted UniFrac methods to generate distance matrices that were later utilized for clustering samples in hierarchical cluster trees with Unweighted Pair Group Method with Arithmetic mean (UPGMA) and Principal Coordinate Analyses representations (PCoA).

4.10. Quantitative PCR for specific bacteria and fungal and bacterial loads

4.10.1. Specific bacteria: *F. prausnitzii, A. muciniphila, E. coli* and *F. nucleatum*

16S rRNA gene copies of *Faecalibacterium prausnitzii, Akkermansia muciniphila, Escherichia coli* and *Fusobacterium nucleatum* were determined by quantitative Real Time Polymerase Chain Reaction (qPCR).

For *F. prausnitzii, A. muciniphila, E. coli and F. nucleatum*, amplification was carried out using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR reaction was performed in a volume of 25 µL using Power SYBR Green PCR Master Mix containing 100 or 250 nM of each primer, respectively, for *F. prausnitzii* and *A. muciniphila*. TaqMan Universal PCR Master Mix (Applied Biosystems), containing 300 nM of each primer and 100 nM of the probe was used for *E. coli* and *F. nucleatum*. The

cycling conditions for the amplification of the 16S rRNA gene fragments were 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C; except for the *A. muciniphila*: 5 min at 95 °C, and 40 cycles of 15 s at 95 °C, 40 s at 60 °C and 30 s at 72 °C with a final extension of 72 °C for 5 min. (Figure 10).

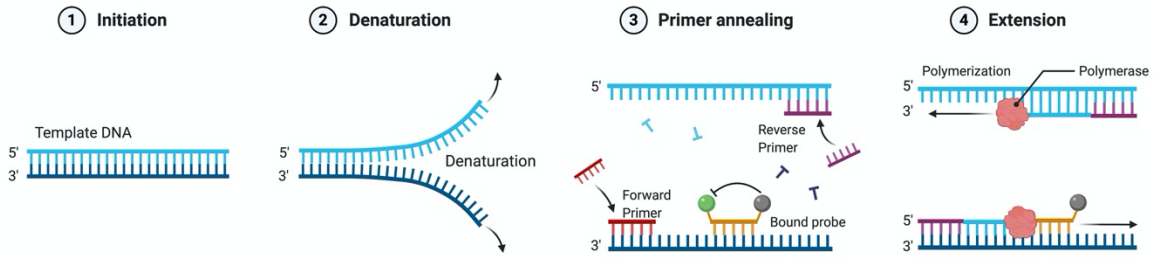

Figure 10. Graphical representation of qPCR amplification. *Created with BioRender.com*

For SYBR Green amplifications, the dissociation step was added to control the specificity of the PCR reaction. All samples were run in triplicate, and mean values were calculated. Data were analyzed using Sequence Detection Software version 1.4 supplied by Applied Biosystems. Specific primers targeting the 16S rRNA gene were used to amplify *F. prausnitzii, A. muciniphila, E. coli,* and *F. nucleatum*.

Table 5. Primers and probes used for targeting specific bacteria

Targets		Nucleotide sequence 5'→ 3'	Size (bp)
Faecalibacterium prausnitzii (222)	Fprau 07 Fprau 02	CCATGAATTGCCTTCAAAACTGTT GAGCCTCAGCGTCAGTTGGT	141
Akkermansia muciniphila (223)	AM1 AM2	CAG CAC GTG AAG GTG GGG AC CCT TGC GGT TGG CTT CAG AT	327
Escherichia coli (224)	Forward Reverse Probe	CATGCCGCGTGTATGAAGAA CGGGTAACGTCAATGAGCAAA FAM-TATTAACTTTACTCCCTTCCTCCCCGCTGAA-TAMRA	96
Fusobacterium nucleatum (225)	Forward Reverse Probe	TTCAATAAAAGTGGCAGGTCAAG TAACAACACATGCAGGTCAATGG FAM-ACTCGAACCCCCAACCCTCGGTTT-TAMRA	100

To generate standard curves, calculated amounts of plasmids, in which the amplified region from each bacterium had been inserted, were used, and serial dilutions were amplified to extrapolate the results.

Plasmid concentration was measured using a NanoDrop ND-1000 Spectrophotometer (ThermoScientific, Wilmington, DE, USA), and the number of copies was calculated from the plasmid molecular weight. Results were expressed in absolute abundance (copies per gram of feces). Undetectable values were replaced with half of the minimal value of the lowest absolute abundance in the data set.

4.10.2. Fungal and bacterial load assessment

For fungal load, the internal transcribed spacer 2 (ITS2) region of fungi was amplified by PCR. Specific primers targeting the ITS2 gene were used for amplification: ITS2-fungi-sense (5'- GTGARTCATCGAATCTTT -3') and ITS2-fungi-antisense (5'-GATATGCTTAAGTTCAGCGGGT -3') (156). PCR was carried out in a volume of 25µL using Power SYBR Green PCR Master Mix containing 300 nM of ITS2 specific primers, following the procedure below: denaturation at 95°C for 2 min, followed by 40 amplification cycles at 95°C for 30 sec, at 55°C for 30 sec, and 72°C for 60 sec, and a final extension cycle at 72°C for 10 min.

To quantify bacterial load, the extracted genomic DNA was used to amplify the V4 region of the 16S rRNA gene by quantitative real-time PCR (qPCR) using the following primers: V4F_517_17 (5'-GCCAGCAGCCGCGGTAA-3') and V4R_805_19 (5'-GACTACCAGGGTATCTAAT-3') (226). The PCR reaction was performed in a total volume of 25 µl using the Power SYBR Green PCR Master Mix containing 100 nM of each of the universal forward and reverse primers. The reaction conditions for amplification of DNA were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate, and mean values were

calculated. Data were analyzed using Sequence Detection Software version 1.4, supplied by Applied Biosystems.

Primers were selected because they matched most of the bacterial sequences deposited in the Ribosomal Database Project (RDP). Plasmid concentration was measured using a NanoDrop ND-1000 Spectrophotometer (ThermoScientific, Wilmington, DE, USA), and the number of copies was calculated from the plasmid molecular weight. Serial dilutions were amplified to extrapolate bacterial (10^2–10^7) and fungal (10^0–10^6) copies number. Results were expressed in absolute abundance (copies per gram of feces). Undetectable values were replaced with half of the minimal value of the lowest absolute abundance in the data set.

4.11. Fecal calprotectin assay

Fecal samples were assayed in a calprotectin enzyme-linked immunosorbent assay (ELISA) (Calprest; Eurospital SpA, Trieste, Italy) according to the manufacturer's instructions. Briefly, stool samples were thawed and 40–120 mg suspended in an extraction solution in a weight per volume ratio of 1:50, mixed by vortex and homogenized. One milliliter of the homogenate was centrifuged for 20 min at 10 000 g, and supernatants were frozen at -20 ºC until analysis. After at least 1:50 dilution in the buffer, supernatants were added to the microtitre plate wells, incubated at room temperature for 45 min and washed three times with washing solution. Purified rabbit anti-human calprotectin antibodies, conjugated with alkaline phosphatase, were added and incubated for 45 min at room temperature. After washing, the enzyme-substrate was added to each well, and 30 min later, the reaction was stopped with 1 M NaOH. Optical densities were read at 405 nm with a microplate ELISA reader (Multiskan EX; Thermo Electron Corporation, Helsinki, Finland). Samples were tested in duplicates, and results were calculated from the standard curve and expressed as μg⁄g stool.

4.12. Statistical analyses

Statistical analyses were carried out in QIIME, R, and GraphPad Prism version 8 (San Diego, CA).
To check the normality of the distribution of the data, we used the Shapiro-Wilk (227) and Anderson-Darling (228) tests. Since the data was not normally distributed, nonparametric tests were used.

We compared quantitative and categorical variables between two unpaired groups with the Mann-Whitney U test (229). When comparing three or more groups, the Kruskal-Wallis test (230) was used. To evaluate categorical variables, we used Fisher's exact test or Chi-square test (231).

To determine the association between quantitative data, we performed Spearman's rank correlation coefficient (232). To test for differences in microbial community composition, we performed analyses with the Adonis test, a nonparametric permutational multivariate analysis of variance (233).

Logistic regression analysis was performed to evaluate the influence of different factors on remission of UC patients at follow-up. For multiple comparisons, we used false discovery rate (FDR) corrected p values to consider significant results sometimes expressed in the text as "q" to avoid the type I error in multiple comparisons (FDR or $q<0.05$ was deemed to be significant). A two-tailed p-value <0.05 was considered to be significant.

5.1. Population description

We recruited one-hundred and eleven individuals (eighty-eight UC patients and twenty-four healthy subjects). We classified UC patients according to previous criteria with twenty-nine patients in the UC long remission group, twenty patients in the UC short remission group, and thirty-eight in the UC flare group. We also included twenty-four healthy controls. Table 6 summarizes the baseline characteristics of UC patients and healthy individuals

Table 6. Demographic and clinical data of the studied groups.

	UC long remission (n=29)	UC short remission (n=20)	UC flare (n=38)	Healthy controls (n=24)	p
Age years (median [Q1-Q3])	41 [34-48.5]	36.5 [33-50]	39 [33.5-44.2]	36.4 [29-43.5]	ns
Female n (%)	13 (44%)	8 (40%)	24 (63%)	14 (58%)	ns
Weight kg (median [Q1-Q3])	76 [67-80]	68 [55.2-78]	63 [54-77.8]	65[56.5-75.7]	<0.05
BMI (median [Q1-Q3])	24.2 [23.6-27.7]	23 [20.2-25.7]	23.1[20.5-26]	23.3 [21.3-25.5]	ns
Current smoker n (%)	4 (13.8%)	4 (22.2%)	5 (13.2%)	6 (25%)	ns
Months since diagnosis (median [Q1-Q3])	204 [143-282]	70.5 [44.5-148]	84[36-133.5]	--	<0.01
Months in remission (median [Q1-Q3])	95[80.2-128.8]	8.65[7.5-11.12]	--	--	<0.01
Montreal classification				--	
A1 / A2 / A3 (n)	2 / 23 / 4	1 / 14 / 5	3 / 26 / 9		ns
E2 / E3 (n)	16 / 13	4 / 16	19 / 19		<0.05
Extraintestinal manifestations	10 (34%)	5 (25%)	5 (14%)		ns
Treatment n (%)				--	
Aminosalycilates	12 (41%)	7 (35%)	25 (65%)		
IMM	7 (24%)	4 (20%)	6 (16%)		
anti-TNF	10 (35%)	9 (45%)	7 (19%)		ns
SCCAI (median [Q1-Q3])	0 [0-1]	0 [0-1]	7.5[7-9]	--	<0.01
Hb g/dL (median [Q1-Q3])	14.2 [12.8-16]	13.9[12.8-15.1]	12.7 [11.5-13.9]	--	<0.01
WBC x10E9/L (median [Q1-Q3])	6.26 [5.84-8.5]	6.52 [5.47-7.76]	7.75 [6.27-9.19]	--	ns

	UC long remission (n=29)	UC short remission (n=20)	UC flare (n=38)	Healthy controls (n=24)	p
Ferritin ng/mL (median [Q1-Q3])	95.5 [38.25-170.5]	52.5[23.5-120.5]	5 [20-77]	--	<0.01
Albumin g/dL (median [Q1-Q3])	4.4 [4.15-4.5]	4.48 [4.35-4.63]	4.1 [3.91-4.41]	--	<0.01
CRP mg/dL (median [Q1-Q3])	0.12 [0.06-0.2]	0.1 [0.04-0.36]	0.5 [0.34-1.17]	--	<0.01
Fecal calprotectin µg/g (median [Q1-Q3])	22 [5-99.8]	56 [11.54-128.8]	825 [347.6-3432]		<0.01

BMI: body mass index; IMM: immunomodulators; SCCAI: Simple Clinical Colitis Activity Index; Hb: hemoglobin; WBC; white blood count; CRP: c-reactive protein.

The median age of UC patients and healthy individuals was thirty-nine and thirty-five years, respectively. Women comprised 52% of UC patients and 58% of controls. Active UC cases, as expected, had significantly higher calprotectin and CRP compared with patients in both short and long remission ($p<0.01$). (Figure 11).
Ferritin, hemoglobin, and albumin were lower in UC flare patients when compared to UC patients in remission ($p<0.01$).

UC long remission patients had a longer disease duration than short remission and flare groups ($p < 0.01$). There was a slightly higher proportion of left-sided extension UC in the UC long remission group than UC short and UC flare disease patients ($p < 0.05$).

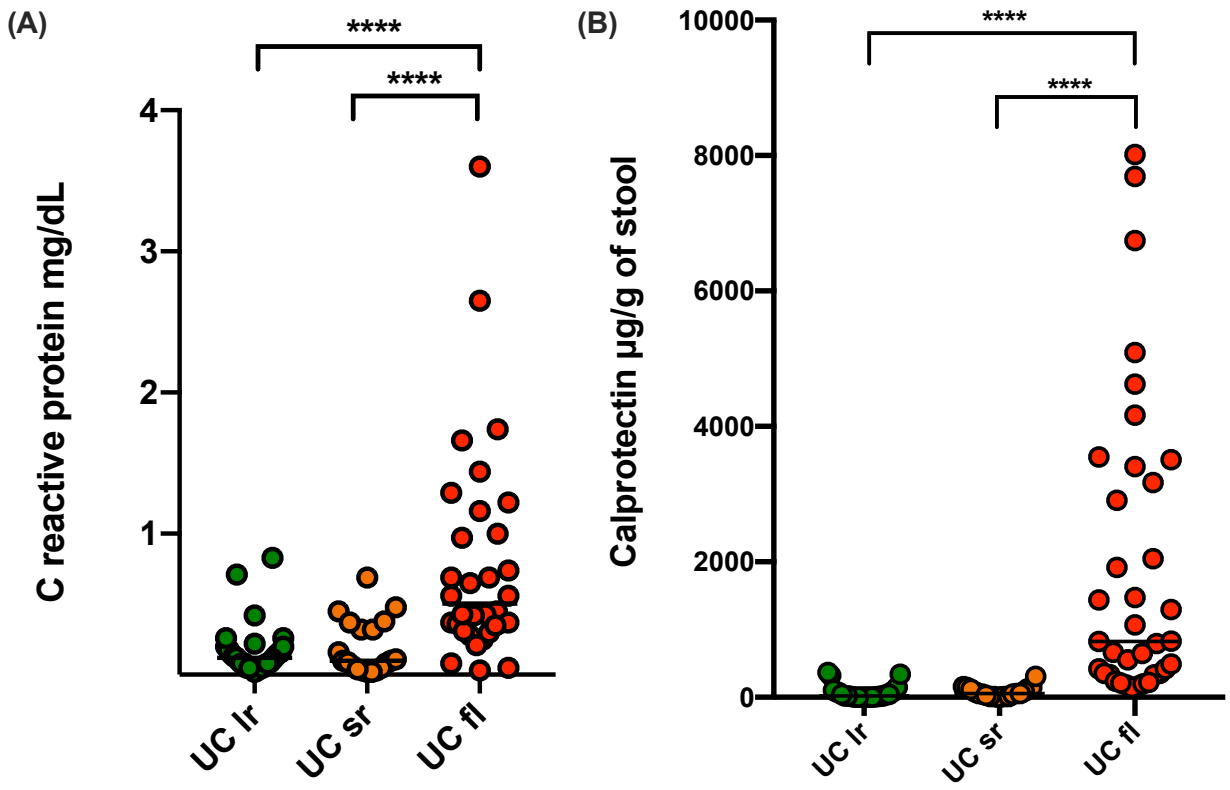

Figure 11. Serologic and fecal biomarkers. UC flare patients (UC fl) presented higher levels of C reactive protein (A) and calprotectin (B) than UC patients in remission (UC lr and UC sr)

5.2. Differences in gut microbiota composition in UC patients and healthy controls using 16S rRNA sequencing technique

Fecal samples from all subjects were subjected to 16S rRNA amplicon sequencing. From a total of 181 fecal samples, after quality filtering we obtained 8,336,717 high quality sequences, with a number of reads ranging from 1,108 to 94,546 per sample. We built the OTU table that contains the taxonomical assignment per OTU and their abundances in each sample.

The number of reads at which we rarefied was 1607, removing samples with less than 1607 reads and we lost one sample from the dataset. We created tables to summarize taxa from Domain to Species levels.

5.2.1. Microbiome stability

We evaluated the stability of the gut microbiota using the unweighted UniFrac distance on UC long and short remission patients' samples and healthy controls at two time points (baseline and week 8). The higher the UniFrac index obtained, the higher the distance between samples. As shown in figure 12, we failed to find any significant differences between time-point samples of UC long remission, UC short remission patients, and healthy individuals (FDR p = 0.2538).

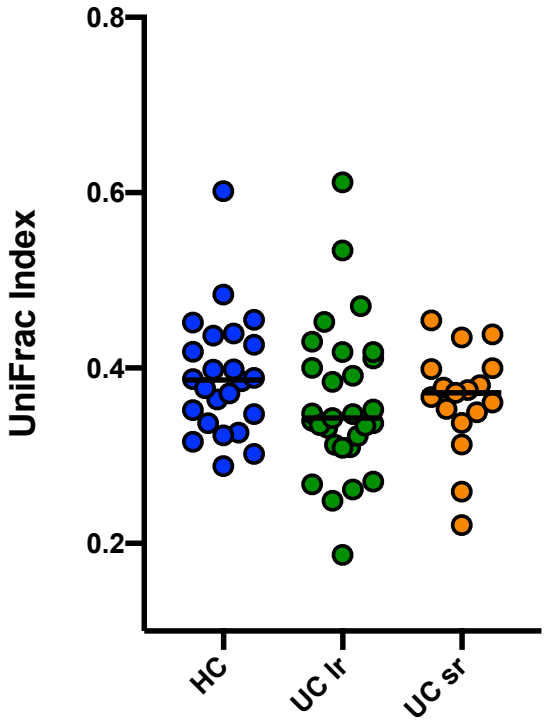

Figure 12. Microbiome stability. Unweighted UniFrac distances calculated between two different time periods for long remission UC patients (UC lr), short remission UC patients (UC sr) and healthy controls (HC). (FDR p = 0.2538)

5.2.2. Species-level operational taxonomic units

We determined the number of observed species-level operational taxonomic units (OTUs) between the different groups. As shown in Figure 13, there were no significant differences in the number of OTUs between healthy individuals and UC long remission patients. However, OTUs number was significantly lower in UC short remission and UC flare patients than healthy controls and UC long remission patients (FDR p <0.0001).

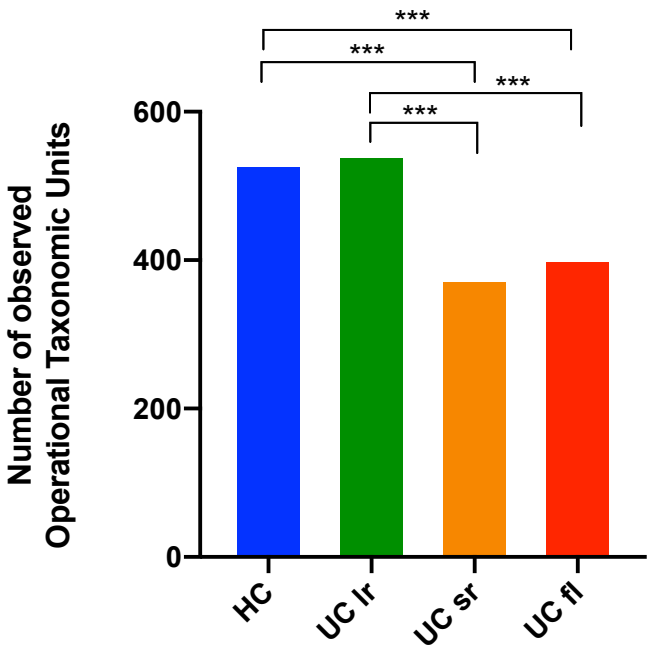

Figure 13. Species-level operational taxonomic units (OTUs). We found a significantly higher number of OTUs in healthy controls (HC) than in short remission (UC sr) and UC flare patients (UC fl). UC long remission patients (UC lr) also showed a significantly higher number of OTUs when compared to UC sr and UC fl patients, while there were no differences in OTUs number between HC and UC lr. ***p<0.001.

5.2.3. Decreased microbial alpha diversity in UC short remission and UC flare patients

Microbial richness was calculated based on the Chao1 index and microbial richness and evenness on the Shannon index. There were some differences found between the different groups (Figure 14); UC short remission and UC flare patients presented significantly lower diversity and evenness than both healthy controls and UC long-remission patients (FDR $p < 0.0001$). However, there were no differences between healthy controls and UC long-remission patients or between short remission and UC flare patients (FDR $p = 0.1038$ and FDR $p = 0.7994$, respectively).

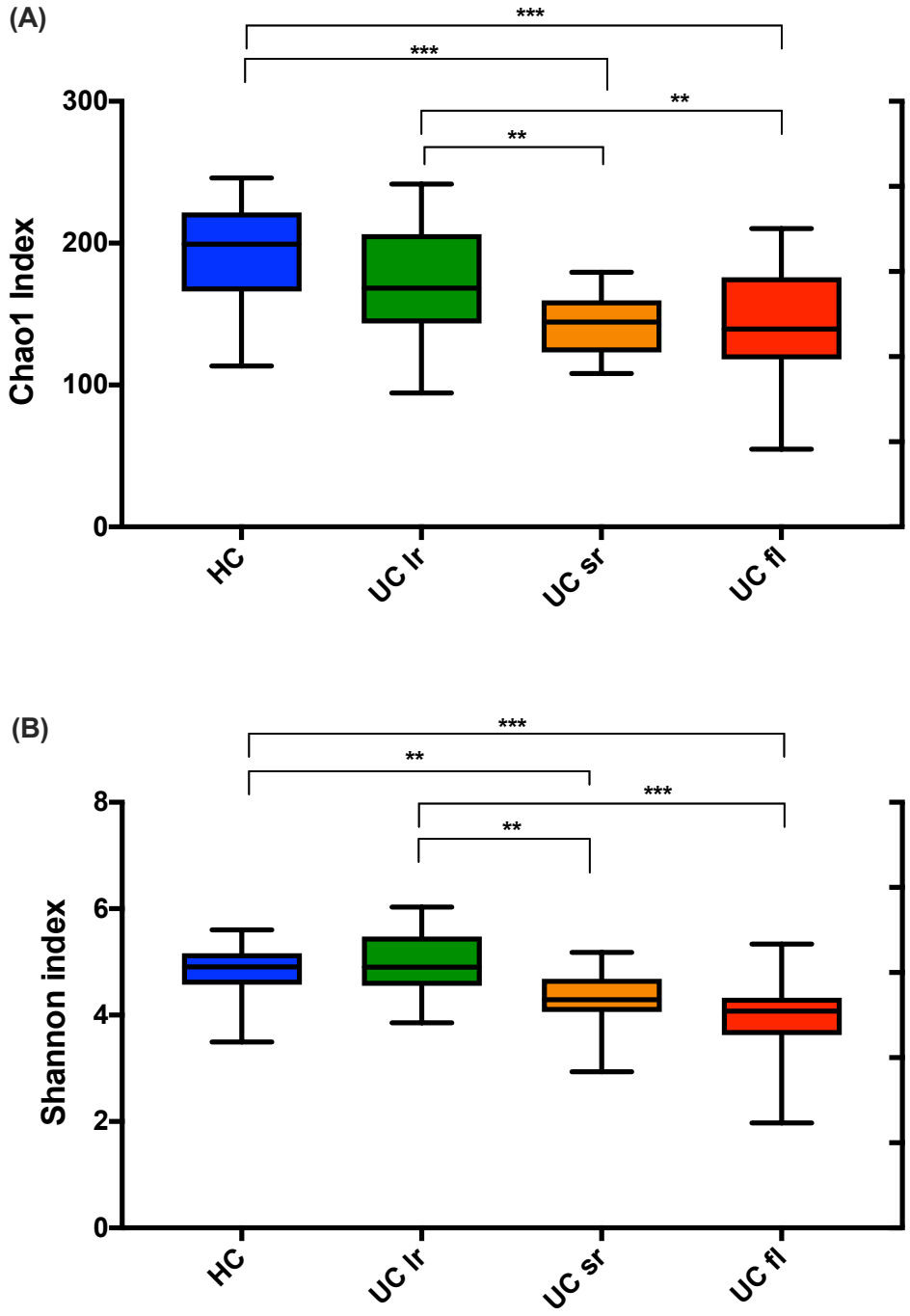

Figure 14. Alpha diversity. Microbial richness was calculated based on the Chao1 index (A) and microbial richness and evenness on the Shannon index (B). Using the Kruskal-Wallis test, UC patients in short remission and flare presented significantly lower richness and evenness than UC patients in long remission and healthy controls. UC patients in long remission and healthy individuals did not present significant differences. **p<0.01; ***p<0.001

5.2.4. Similar gut microbiome beta diversity between UC long remission patients and healthy controls

An unweighted and weighted UniFrac-based Principal Coordinate Analysis (PCoA) analysis of the cohort was performed to visually explore the similarity and variation between samples' microbial composition and abundance (Figure 15).

UniFrac is a metric used for comparing microbial community composition between samples. As mentioned previously, unweighted UniFrac takes into consideration only the microbial composition, whereas weighted UniFrac considers the microbial composition and microbial abundance. Unweighted UniFrac showed that healthy controls and UC long-remission predominantly clustered on the right side of the plot, and UC active disease and short-remission samples positioned on the left, although there is an overlap of some samples, especially UC-long remission to the left. (Figure 15A).

Pairwise, Adonis was performed for beta diversity. We found significant differences between the groups in the following comparisons: healthy individuals and UC short-remission and UC flare patients (FDR $p = 0.001$ for unweighted and FDR $p = 0.02$ for weighted UniFrac). Between UC long remission and UC short-remission (FDR $p = 0.001$ for unweighted and FDR $p = 0.043$ for weighted UniFrac) and between UC long remission and UC flare patients (FDR $p = 0.001$ for unweighted and weighted UniFrac). Only significant differences with unweighted UniFrac were observed between Healthy controls and UC long remission (FDR $p = 0.032$).

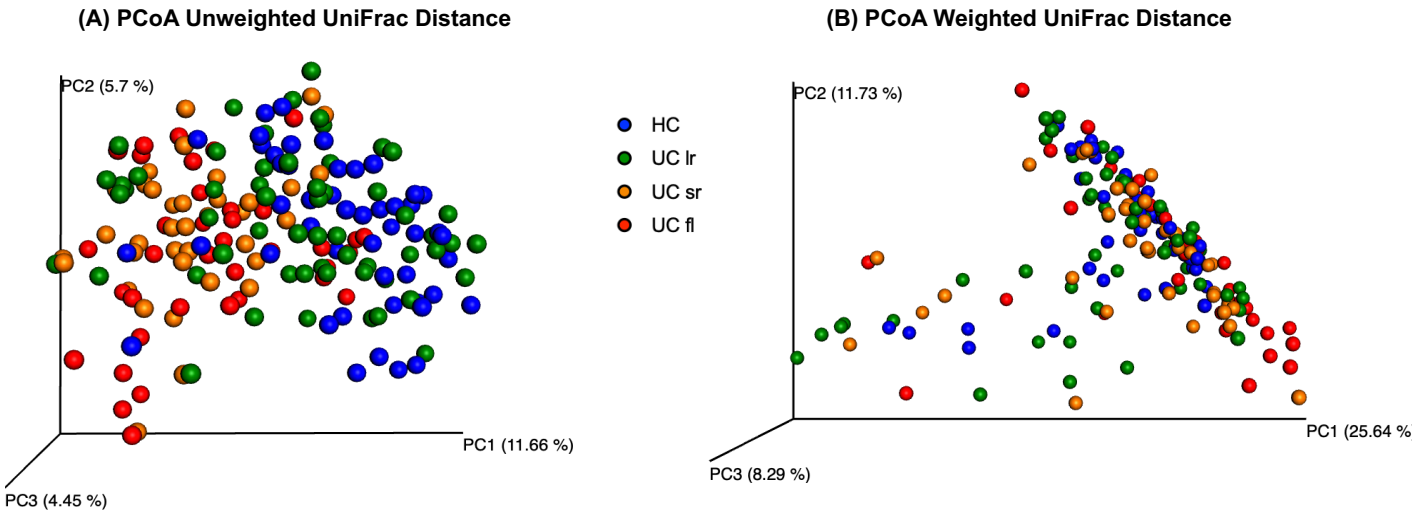

Figure 15. Principal Coordinates Analysis of unweighted (A) and weighted (B) UniFrac distances. In unweighted UniFrac, there were significant differences observed between healthy controls (HC) and UC patients in short remission (UC sr) (FDR p=0.001), HC and UC flare patients (UC fl) (FDR p=0.001), and HC and UC long remission patients (UC lr) (FDR p=0.032). Differences were also found between UC lr and UC sr (FDR p=0.001) and UC lr and UC fl (FDR p=0.001). In weighted UniFrac, there were significant differences between HC and UC sr (FDR p=0.022), HC and UC fl (FDR p=0.012), and also between UC lr and UC sr (FDR p=0.001) and UC lr and UC fl (FDR p=0.001). No differences were observed with weighted UniFrac between HC and UC lr.

5.2.5. Differences in bacterial taxa between UC patient groups and healthy individuals

At the phyla level, Firmicutes and Bacteroidetes comprised more than 90% of bacteria in healthy controls and UC patients, as seen in Figure 16.

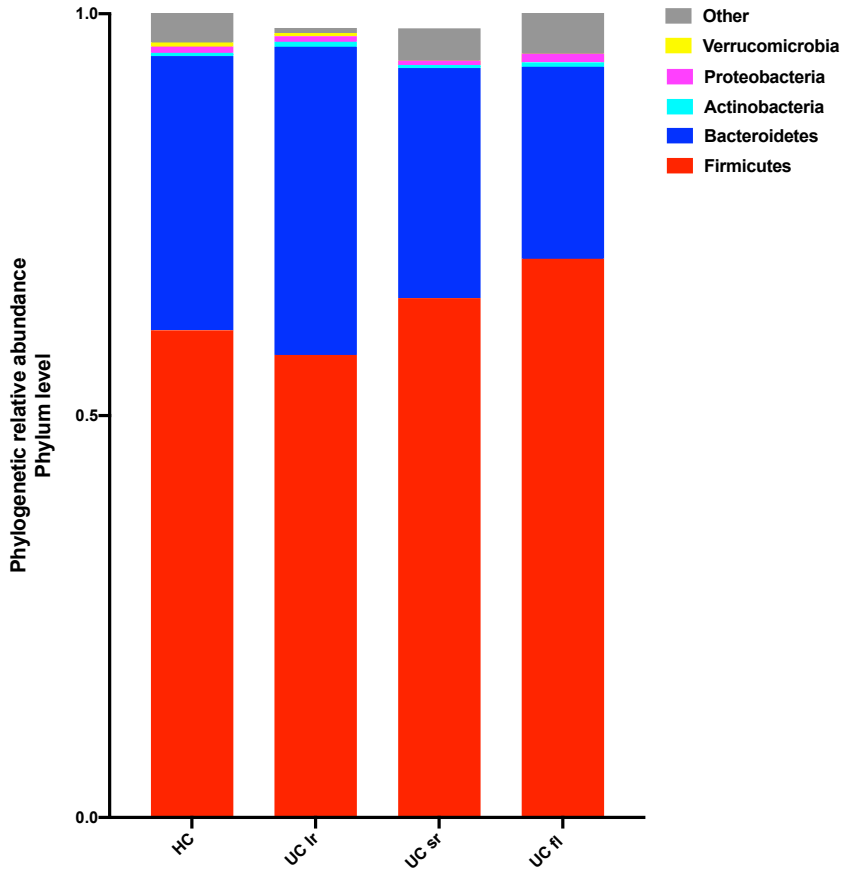

Figure 16 Global composition of bacterial microbiota at the phyla level.

There was a lower relative abundance of Bacteroidetes phyla in UC flare patients than healthy individuals (FDR p = 0.0478) and UC long remission patients (FDR p = 0.0243). Healthy controls and UC long remission patients had a significantly higher relative abundance of Verrucomicrobia than UC short remission (FDR p = 0.0004 and FDR p = 0.004, respectively) and UC flare patients (FDR p = 0.0009 and FDR p = 0.0159 respectively). No differences in Verrucomicrobia abundance were found between healthy controls and UC long remission patients, and between UC short remission and UC flare patients. An unknown phylum was significantly lower in abundance in UC long remission compared to healthy controls and UC short remission and flare patients (FDR p < 0.0001, FDR p = 0.0009, and FDR p < 0.0001, respectively). (Figure 17).

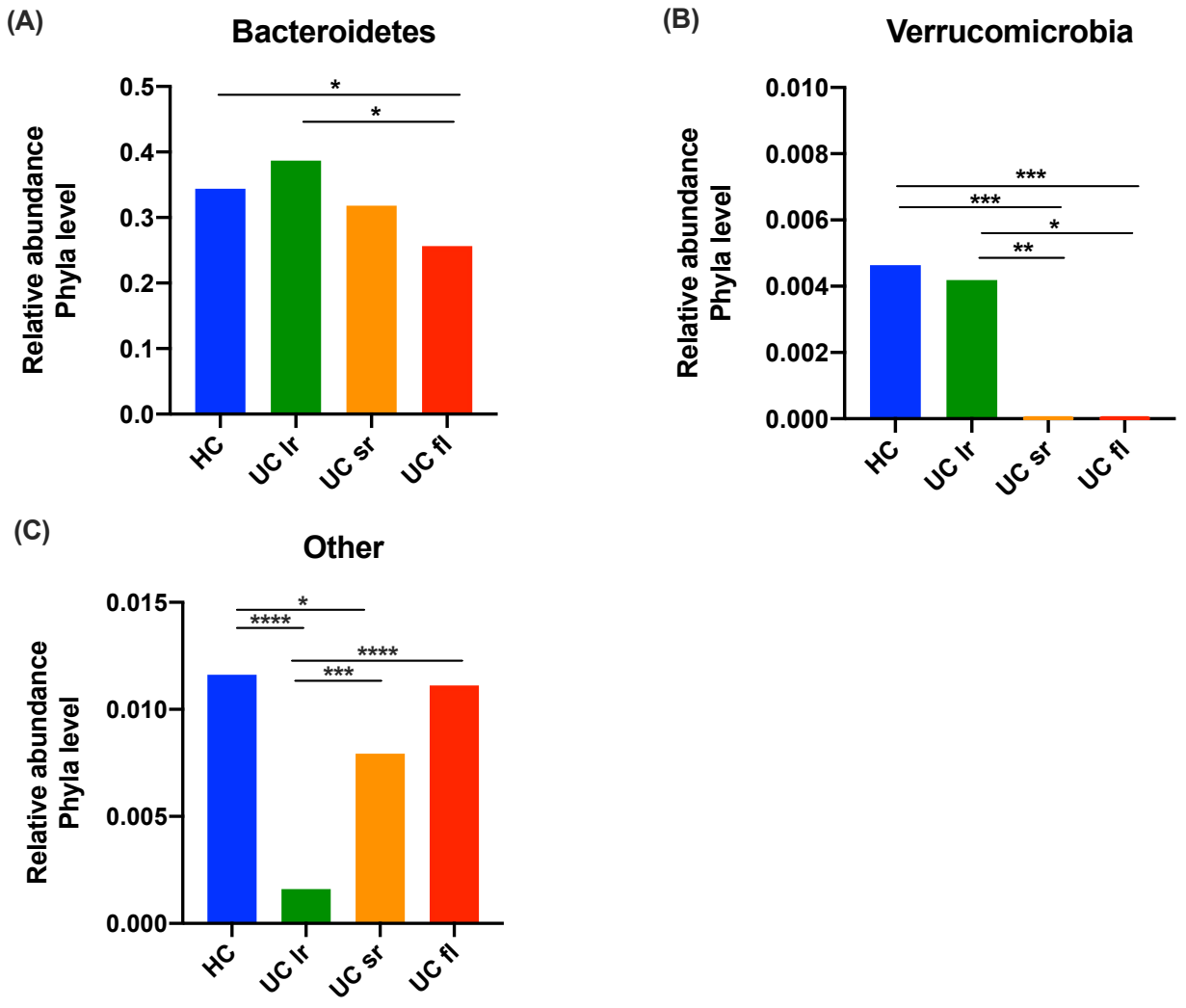

Figure 17. Differences at the phyla level. Bacteroidetes (A) relative abundance was lower in UC flare patients (UC fl) than in healthy subjects (HC) and UC long remission patients (UC lr). The abundance of Verrucomicrobia (B) was significantly higher in HC and UC lr when compared to both UC short remission (uC sr) and UC fl. An unidentified phylum was significantly lower in UC lr than in the other group subjects. *p<0.05, **p<0.01; ***p<0.001.

We identified several genera significantly different between healthy controls, UC long remission, UC short remission, and UC flare patients at the genus level. (Figure 18). Notably, genus *Akkermansia* showed a higher abundance in Healthy controls and UC long remission than in UC short remission and UC flare patients (FDR p = 0.0003 and FDR p = 0.0019, respectively). Likewise, healthy subjects and UC long remission patients had a higher relative abundance of *Paraprevotella* than UC short remission and UC flare patients (FDR p = 0.0002 and FDR p <0.0001, respectively).

Coprococcus abundance was significantly higher in healthy controls when compared to all UC patients; UC long remission (FDR p = 0.0143), UC short remission (FDR p = 0.0143), and UC flare (FDR p = 0.0004). In contrast, genus *Faecalibacterium* relative abundance was not significantly different between groups (FDR p = 0.1233).

Collinsella relative abundance was higher in UC flare patients when compared to healthy controls (FDR p = 0.0127), UC long remission (FDR p = 0.0012), and UC short remission patients (FDR p = 0.0089), while *Peptostreptococcus* abundance was also higher in UC flare patients than in all other groups; Healthy controls (FDR p = 0.0064), UC long remission (FDR p = 0.0064), and UC short remission patients (FDR p = 0.0064). At the same time, *Lachnospira* had a reduced abundance in UC flare patients compared to UC long (FDR p = 0.0014), and short remission patients (FDR p = 0.0070).

Streptococcus genera was more abundant in UC short remission and UC flare than healthy controls (FDR p = 0.0020 and FDR p = 0.0176 respectively), and in UC short remission when compared to UC long remission patients (FDR p =0.0074). *Eggerthella* abundance was lower in UC long remission than UC short remission (FDR p = 0.0154).

Both *Blautia* and *Dorea* genera relative abundance was significantly higher in UC short remission patients than in all other subjects; Healthy controls (FDR p = 0.0021 and FDR p = 0.0013, respectively), UC long remission (FDR p = 0.0006 and FDR p = 0.0171, respectively), and UC short remission patients (FDR p = 0.0006 and FDR p = 0.0002, respectively).

Healthy controls and UC long remission patients showed a higher abundance of *Parabacteroides* genera than in UC flare patients (FDR p = 0.0434 and FDR p = 0.0001, respectively) and in UC long remission when compared to UC short remission patients (FDR p = 0.0434).

UC long remission patients showed a higher abundance of the genus *Alistipes* than in all other subjects; Healthy controls (FDR p = 0.0018), UC short remission (FDR p = 0.0128), and UC flare patients (FDR p = 0.0003). Likewise, an unknown genus of the *Rikenellaceae* family was more abundant in UC long remission than in UC short remission and UC flare patients (FDR p = 0.0038 and FDR p = 0.0014).

Finally, *Ruminococcus* and *Oxalobacter* genera showed a higher abundance in healthy controls than in UC short remission (FDR p = 0.0015 and FDR p = 0.0021, respectively), and UC flare patients (FDR p = 0.0183 and FDR p = 0.0018, respectively).

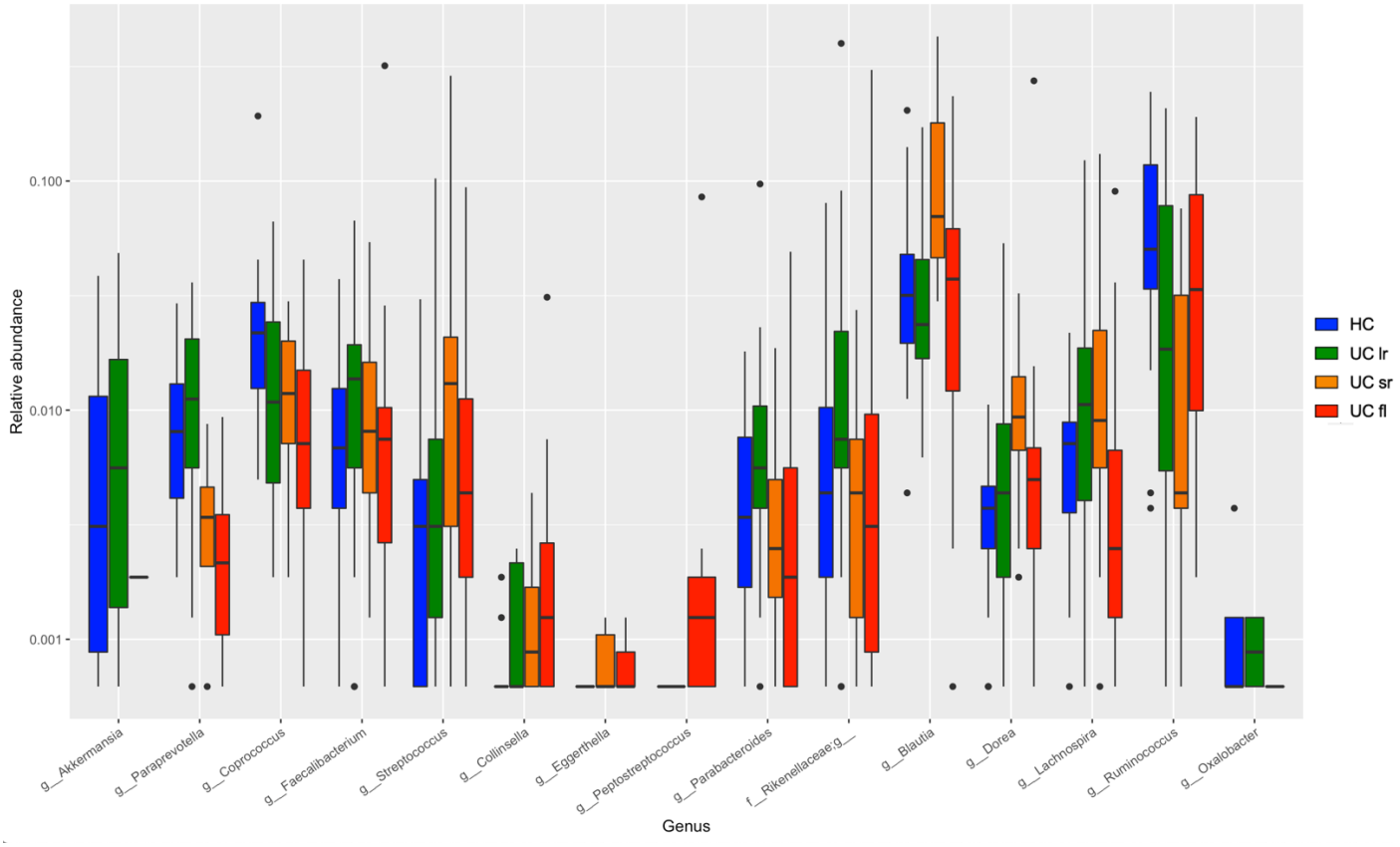

Figure 18. Relative abundance of bacteria at genus level. Significant taxa relative abundance between healthy controls (HC), UC patients in long remission (UC lr), UC short remission (UC sr), and UC flare patients (UC fl).

As seen in Table 7, we found sixty-seven species significantly different between the four groups at the species level.

Table 7. List of species that were differentially abundant between Healthy controls, UC long remission patients, UC short remission patients, and UC flare patients. FDR column corresponds to FDR correction for Kruskal-Wallis test between the four groups. Values represent the difference in relative abundance (relative proportion over a total of 1.0) for each species between both groups. In blue, species enriched in healthy controls (HC), in green, species enriched in UC long remission (UC lr), in orange, species enriched in UC short remission (UC sr), and in red, species enriched in UC flare UC fl). Blank spaces indicate that those species are not significantly different in that comparison.

Species	HC vs UClr	HC vs UCsr	HC vs UCfl	UClr vs UCsr	UClr vs UCfl	UCsr vs UCfl	FDR p
f__Lachnospiraceae;g__;s__183	0.00074924	0.0012653	0.00142255	0.00051606	0.00067331		<0.0001
f__Lachnospiraceae;g__;s__66		0.00900747	0.00902513	0.00676244	0.0067801		<0.0001
f__Ruminococcaceae;g__;s__54		0.01230554	0.0121281	0.00863357	0.00845613		<0.0001
f__Ruminococcaceae;g__Oscillospira;s__69		0.00900228	0.00638115	0.01078149	0.00816036		<0.0001
f__Ruminococcaceae;g__;s__81		0.00077785	0.0006433	0.00587945	0.0057449		<0.0001
f__Rikenellaceae;g__;s__275	-0.0001735		0.00189346	0.00165655	0.00206691	0.00041037	<0.0001
f__Christensenellaceae;g__;s__158	0.00195177	0.00298175	0.00288084				<0.0001
f__Ruminococcaceae;g__;s__172	0.00016719	0.00121863	0.00121863	0.00105143	0.00105143		<0.0001
f__Lachnospiraceae;g__;s__163	0.00590538	0.00618648	0.00618143				0.0001
f__Lachnospiraceae;g__Blautia;s__1		-0.0486154		-0.0553827		0.05141358	0.0001
g__Coprococcus;s__eutactus	0.00850982	0.00984236	0.01002231				0.0001
g__Ruminococcus;s__bromii	0.03098871	0.05761253	0.03782158	0.02662382			0.0001
g__Paraprevotella;s__74		0.00462041	0.00501396	0.00564771	0.00604125		0.0002
o__Clostridiales;f__;g__;s__229		0.00088156	0.00074701	0.00087977	0.00074523		0.0002
f__Rikenellaceae;g__;s__93		0.00147791		0.0042272	0.00350401		0.0002
g__Bacteroides;s__38		0.00532566	0.00344285	0.00635474	0.00447193		0.0003
f__Ruminococcaceae;g__;s__251	0.00094772	0.00210537	0.00191449	0.00115765			0.0003
g__Lachnospira;s__126			0.00079747		0.00470101	0.00293396	0.0004
g__Akkermansia;s__muciniphila		0.00438187	0.00127048	0.00416282	0.00105144		0.0007
g__Oxalobacter;s__formigenes	0.00026018	0.00038892	0.00037211				0.001
o__Clostridiales;f__;g__;s__197			0.00107777		0.00199152		0.0012
g__Peptostreptococcus;s__anaerobius			-0.0025732		-0.0025517	-0.0025732	0.0012
f__Ruminococcaceae;g__;s__157		0.00157125	0.00055711	0.00195481	0.00094067		0.0012
g__Collinsella;s__179			-0.0017989		-0.0018141	-0.0016692	0.0014
f__Ruminococcaceae;g__;s__111			0.00107427	0.00498573	0.00511103		0.002
f__Christensenellaceae;g__;s__618	0.00015557	0.00015557	0.00015557				0.0021
g__Desulfovibrio;s__D168	0.00095934	0.00095934	0.00095934				0.0021
g__Parabacteroides;s__distasonis					0.00049469		0.0031
f__Ruminococcaceae;g__;s__363			-0.0014064		-0.0015218	-0.0012353	0.0031
f__Coriobacteriaceae;g__;s__231		0.00147272	0.00147441				0.0033
g__Lachnospira;s__37					0.0061671	0.00962932	0.0034
o__Clostridiales;f__;g__;s__429		0.00028003	0.00029432				0.004

Species	HC vs UClr	HC vs UCsr	HC vs UCfl	UClr vs UCsr	UClr vs UCfl	UCsr vs UCfl	FDR p
g__Alistipes;s__indistinctus	-0.000089408			0.00042916	0.00039552		0.0042
f__Ruminococcaceae;g__;s__342	0.00067056	0.00071562	0.00071057				0.0042
g__Streptococcus;s__32		-0.025446	-0.0041674	-0.0224535			0.0043
o__Clostridiales;f__;g__;s__280		0.0007156	0.00048773				0.0044
f__Ruminococcaceae;g__;s__34		0.01149658	0.00722557	0.01545287	0.01118186		0.0053
g__Dialister;s__424			-0.0002355		-0.0002355	-0.0002355	0.0061
g__Parabacteroides;s__78					0.00193121		0.0064
f__Lachnospiraceae;g__;s__225		0.000363		0.00053645	0.0001531		0.0068
g__Dorea;s__31		-0.0043248		-0.0035577		0.0026169	0.0085
g__Bacteroides;s__caccae				0.00115551	0.0020124		0.0089
f__Ruminococcaceae;g__;s__182				0.00176169	0.00076436		0.0105
g__Paraprevotella;s__153		0.0003267	0.000086194	0.00156857	0.00132807		0.0113
f__Christensenellaceae;g__;s__328		0.00062228	0.00062228				0.0113
o__Clostridiales;f__;g__;s__496				0.00034333	0.00034333		0.0114
f__Lachnospiraceae;g__;s__123					0.00081192		0.0115
f__Ruminococcaceae;g__;s__186	0.00031114	0.00031114	0.00031114				0.012
g__Bacteroides;s__uniformis			0.03511033				0.0123
f__Lachnospiraceae;g__;s__19			0.00598171				0.0132
g__Eggerthella;s__lenta				-0.0002489			0.0137
f__Ruminococcaceae;g__;s__67			0.0014254		0.001817		0.0137
g__Lachnospira;s__73			0.0023658		0.00081714		0.0156
g__Bulleidia;s__moorei			-0.0002355		-0.0002355	-0.0002355	0.0167
f__Ruminococcaceae;g__;s__84					0.0016134		0.0177
f__Ruminococcaceae;g__;s__515		0.00023336	0.000098808				0.0195
g__Alistipes;s__massiliensis					0.00017166		0.0201
f__Rikenellaceae;g__;s__53					0.0070399		0.0241
f__Ruminococcaceae;g__;s__316				0.00023604	0.00021922		0.0243
f__Ruminococcaceae;g__;s__419		0.00020743	0.00019061				0.0258
g__Bacteroides;s__109					0.01841199		0.0259
o__Clostridiales;f__;g__;s__164					0.0008954		0.0298
f__Ruminococcaceae;g__;s__104			0.00491095				0.0314
o__Clostridiales;f__;g__;s__175			0.00081429				0.0349
g__Coprococcus;s__52			0.00641338				0.0365
g__Blautia;s__146			0.00173929				0.0449
g__Ruminococcus;s__409					0.00083164		0.0465

As shown in Figure 19, fourteen species differed between healthy individuals and UC long remission patients at baseline, twelve of these were enriched in healthy controls, and the two remaining species were more abundant in UC long remission patients.

When comparing healthy and UC short remission, thirty-four species differed in abundance between these groups, with thirty-one having a higher relative abundance in

HC. Moreover, forty-seven species identified as having a different abundance between healthy subjects and UC flare patients, forty-one of which were more abundant in healthy controls.

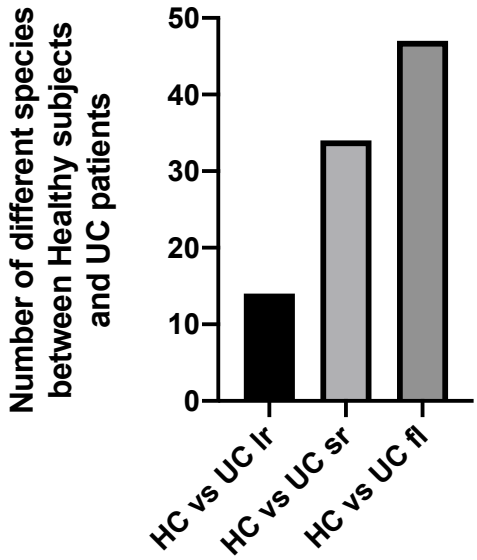

Figure 19. Number of different species between healthy subjects and UC patients. Healthy control (HC) *vs* UC long remission (UC lr), UC short remission (UC sr), and UC flare (UC fl).

Between the different UC groups, we identified twenty-eight species that were significantly different between UC long remission and UC short remission patients, with three species having a higher abundance in short remission. Between UC long remission and flare patients, forty-two species were significantly different, with five species being significantly enriched in flare patients. In contrast, we found only ten significant species that differed between UC short remission and UC flare patients, half of which were enriched in short remission and the other half more abundant in UC flare patients. (Figure 20).

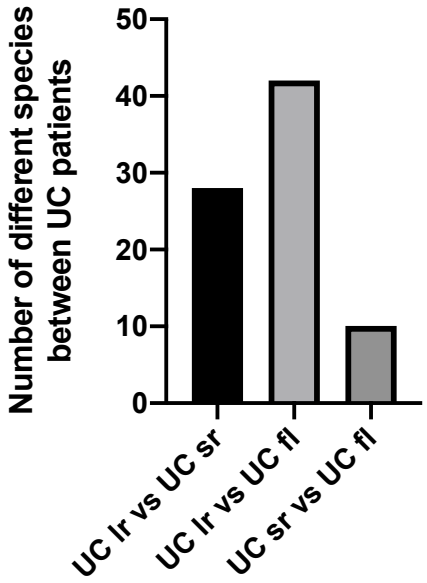

Figure 20. Number of different species between UC patients. UC long remission (UC lr) *vs* UC short remission (UC sr), and UC flare (UC fl)

5.3. Differences in specific species between healthy controls and UC patients' groups by qPCR results

To further analyze the samples, we investigated specific bacteria associated with UC in other studies. For this purpose, we explored the absolute abundance, i.e., copies per gram of feces of specific bacteria with qPCR. Results were similar to those of the 16S rRNA amplicon sequencing data.

Akkermansia muciniphila showed a higher absolute abundance in healthy controls (median 3.32E+09 copies/g of feces) and UC long remission patients (median 3.56E+06 copies/g of feces) than in UC short remission (median 1.12E+05 copies/g of feces, FDR p = 0.0002 and FDR p = 0.0061, respectively) and UC flare patients (median 1.12E+05 copies/g of feces, FDR p = 0.0005 and FDR p = 0.0275, respectively). (Figure 21A).

In contrast, the absolute abundance of *Faecalibacterium prausnitzii* was not significantly different between the groups. However, there was a trend for *F. prausnitzii* abundance to be higher in healthy controls (median 1.20E+09 copies/g of feces) and UC long remission patients (median 1.59E+09 copies/g of feces) when compared to UC short remission (median 4.44E+08 copies/g of feces, FDR p = 0.12 and FDR p = 0.082, respectively) and UC flare patients (median 5.40E+08 copies/g of feces, FDR p = 0.073 and FDR p = 0.081, respectively)

As shown in Figure 21, the absolute abundance of *Escherichia coli* (Healthy controls (median 3.18E+08 copies/g of feces), UC long remission patients (median 1.30E+08 copies/g of feces), UC short remission (median 1.18E+08 copies/g of feces) and UC flare patients (median 8.63E+07 copies/g of feces), and *Fusobacterium nucleatum* (Healthy controls (median 5.63E+05 copies/g of feces), UC long remission patients (median 2.30E+05 copies/g of feces), UC short remission (median 3.66E+05 copies/g of feces) and UC flare patients (median 5.48E+05 copies/g of feces), was similar across all groups.

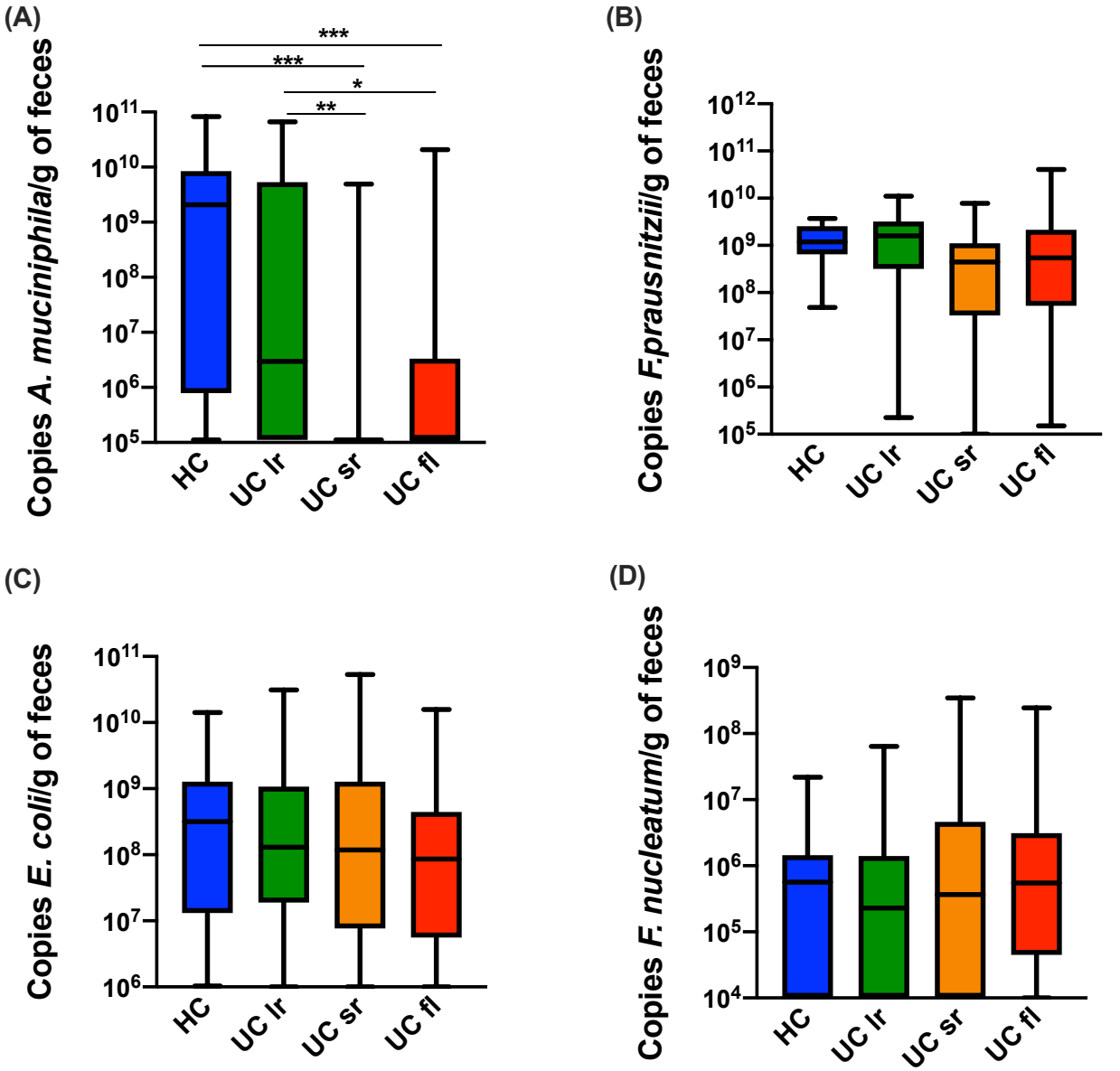

Figure 21. Absolute abundance of bacteria by qPCR. *Akkermansia muciniphila* (A) had a higher abundance in healthy controls (HC) and UC patients in long remission (UC lr) than in UC short remission (UC sr), and UC flare patients (UC fl). The absolute abundance of *Faecalibacterium prausnitzii* (B), *Escherichia coli* (C), and *Fusobacterium nucleatum* (D) was not significantly different between all groups. *p<0.05, **p<0.01; ***p<0.001.

5.4. Relationship between microbiota and clinical data of UC patients

We explored the association of microbiota findings and clinical data of UC patients. We found no differences between microbiota data such as species-level number of OTUs, microbial diversity, and specific bacteria and patient variables such as BMI, weight, gender, age, UC extension, calprotectin, CRP, or albumin. There were also no significant differences in microbiota composition when comparing the different patient treatments.

However, the Chao1 index was significantly decreased in current smokers when compared to never smokers (FDR $p = 0.0068$) and former smokers (FDR $p = 0.01$). (Figure 22A).

In contrast, the Shannon index (Figure 22B) showed no differences between smokers and non-smokers. Furthermore, there were no differences found in bacteria within the smoking status at the phyla and genus levels.

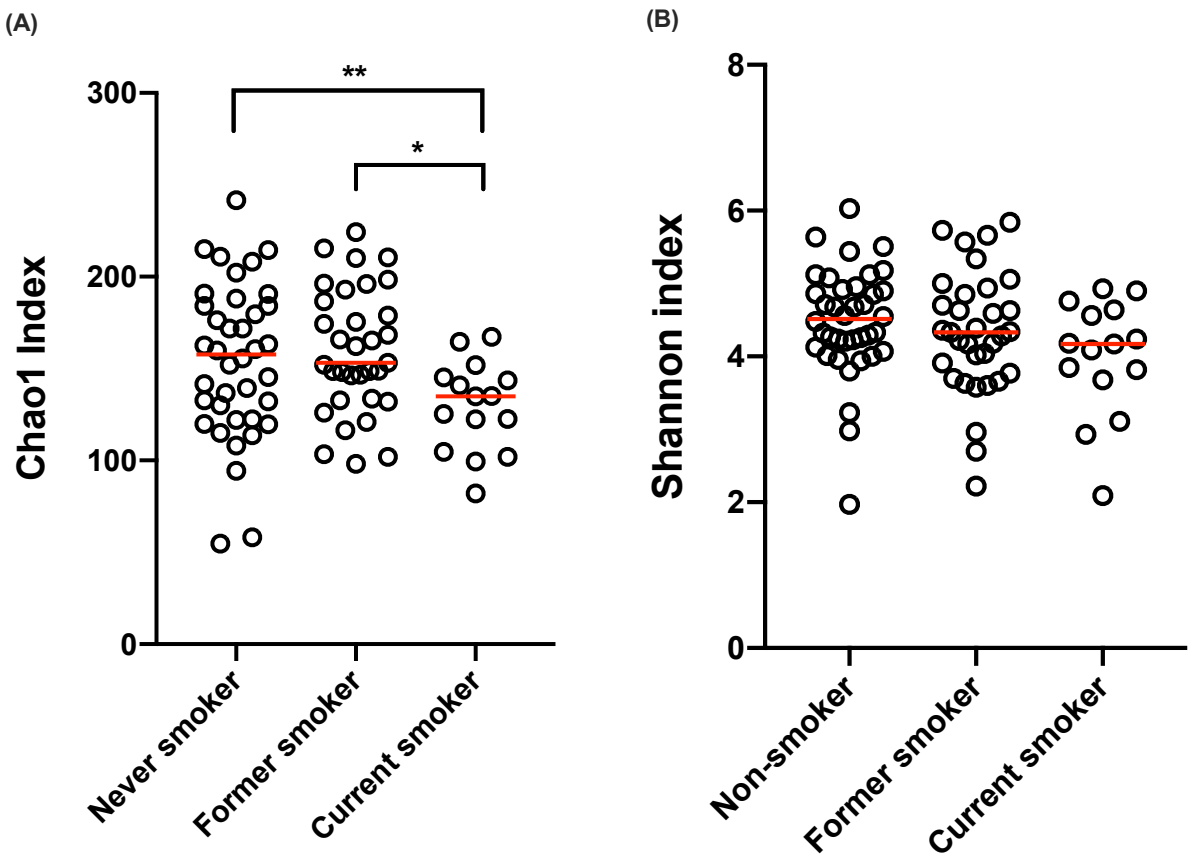

Figure 22. Smoking status and alpha diversity. Chao1 index (A) was significantly lower in current smokers than in non-smokers and former smokers. There were no differences in the Shannon index (B). *p<0.05, **p<0.01.

We ran a Spearman's correlation test to assess the relationship between *Akkermansia muciniphila* absolute abundance and months in remission in UC. We included all samples from UC patients in long and short remission. (Figure 23). There was a moderate positive correlation between *A. muciniphila* absolute abundance and time in remission, which was statistically significant ($r_s = 0.53$, $p < 0.001$)

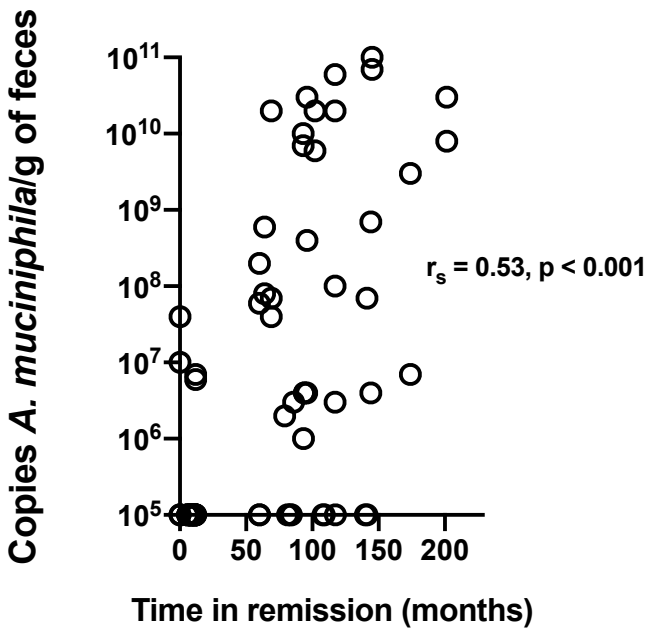

Figure 23. Correlation between absolute abundance of *Akkermansia muciniphila* and time in remission. We found a moderate positive correlation between *A.muciniphila* absolute abundance and time in remission ($r_s = 0.53$, $p < 0.001$)

5.5. Follow-up cohort of UC patients

UC long remission and short remission patients (n=49) were further followed for 24 months after inclusion to determine the clinical activity of the disease. Fifteen patients presented a flare of UC, with 12 patients requiring oral corticosteroids to treat their flare. Nine patients were lost to follow-up.

We examined the association of baseline microbiota variables such as the Chao1 index, the Shannon index, and the presence of *Akkermansia muciniphila* with flare outcome. Univariate analysis showed that the Shannon index and presence of *A. muciniphila* were associated with clinical remission.

We performed a logistic regression analysis to ascertain the effects of the Shannon index and the presence of *A. muciniphila* on the likelihood that patients had a flare-free outcome at follow-up. A greater Shannon index (OR: 4.83; p 95% CI=1.5-20.6) and presence of *A. muciniphila* (OR: 4.9; p 95% CI=1.12-29.08) were independently associated with clinical remission. The model was able to classify flare-free outcomes in 84% of cases correctly.

We ran a log-rank test to determine if there were differences in the flare-free distribution for the presence or absence of *A. muciniphila*. The distributions were significantly different, with the presence of *A. muciniphila* associated with clinical remission at 24 months ($\chi 2 = 4.1$, $p = 0.0428$) (Figure 24).

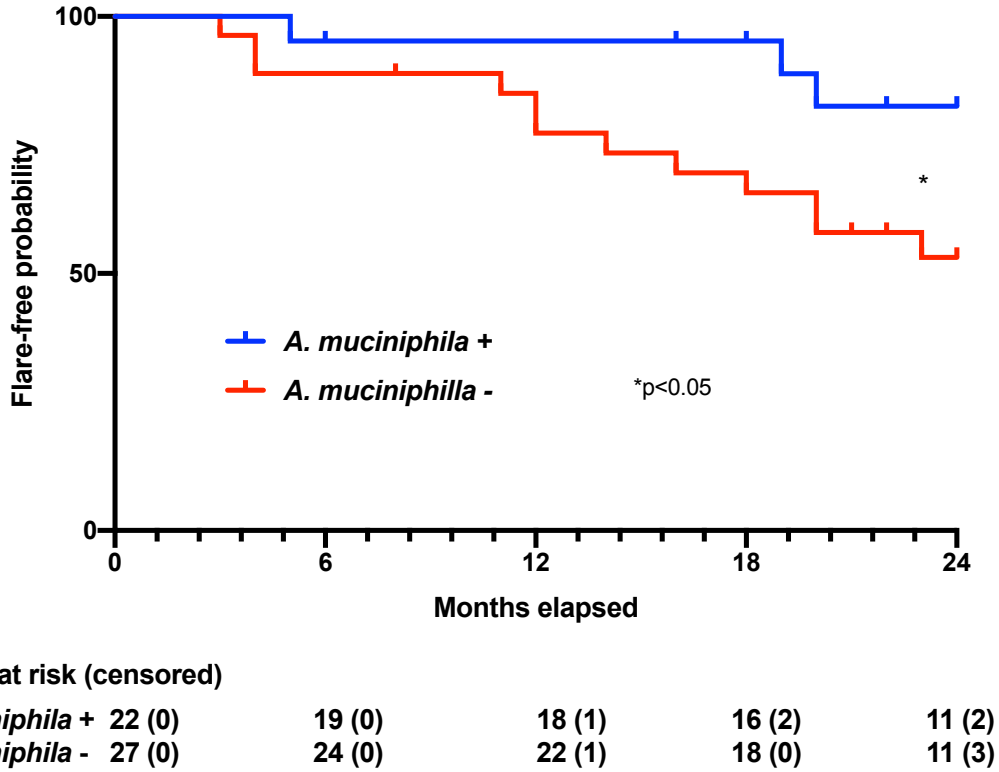

Figure 22. Kaplan-Meier curve on flare-free outcome of Ulcerative colitis patients (n=49) with presence of *Akkermansia muciniphila* (n = 22) and absence of *A. muciniphila* (n=27). The overall flare-free outcome for patients with presence of *A. muciniphila* was significantly better than that for patients without *A. muciniphila* (log rank test: p <0.05)

5.6. Fungal and bacterial load in healthy subjects and UC patients' groups

5.6.1. Characterization of fungal and bacterial load in healthy individuals

We explored the fungal (ITS2 gene) and bacterial (16srRNA gene) load in healthy individuals. As shown in Figure 25, fecal samples contained a significantly lower number of ITS2 gene copies (median 1.53E+06 copies/g of feces [Q1-Q3: 3.85E+05 - 9.61E+06]) than 16srRNA gene copies (median 9.93E+11 copies/g of feces [Q1-Q3: 6.00E+11 - 1.38E+12]) ($p < 0.0001$).

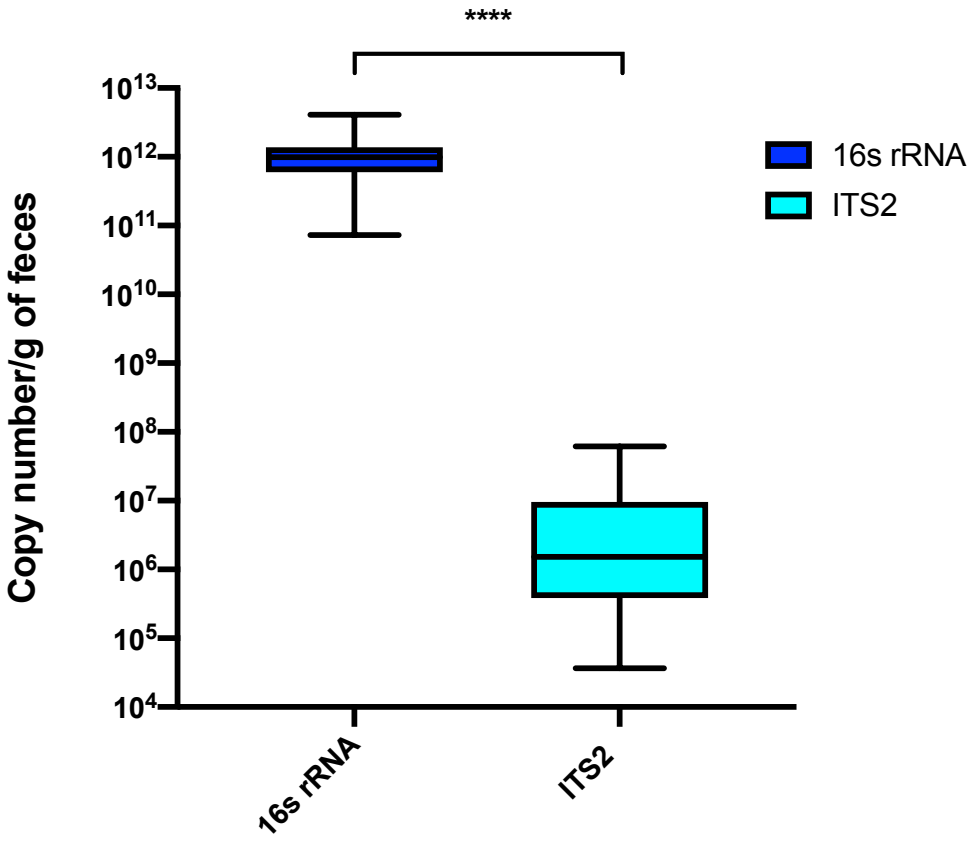

Figure 23. Fungal (ITS2 gene) and bacterial (16S rRNA gene) loads. Fungal load was significantly lower in fecal samples than the bacterial load in healthy individuals. ****p<0.0001

We analyzed the variation between samples over time. We compared the number of copies of the ITS2 and 16s rRNA genes in fecal samples collected at two different time points: basal versus eight-week samples.

The overall fungal and bacterial loads did not show any significant variations during these two periods. (Figure 26).

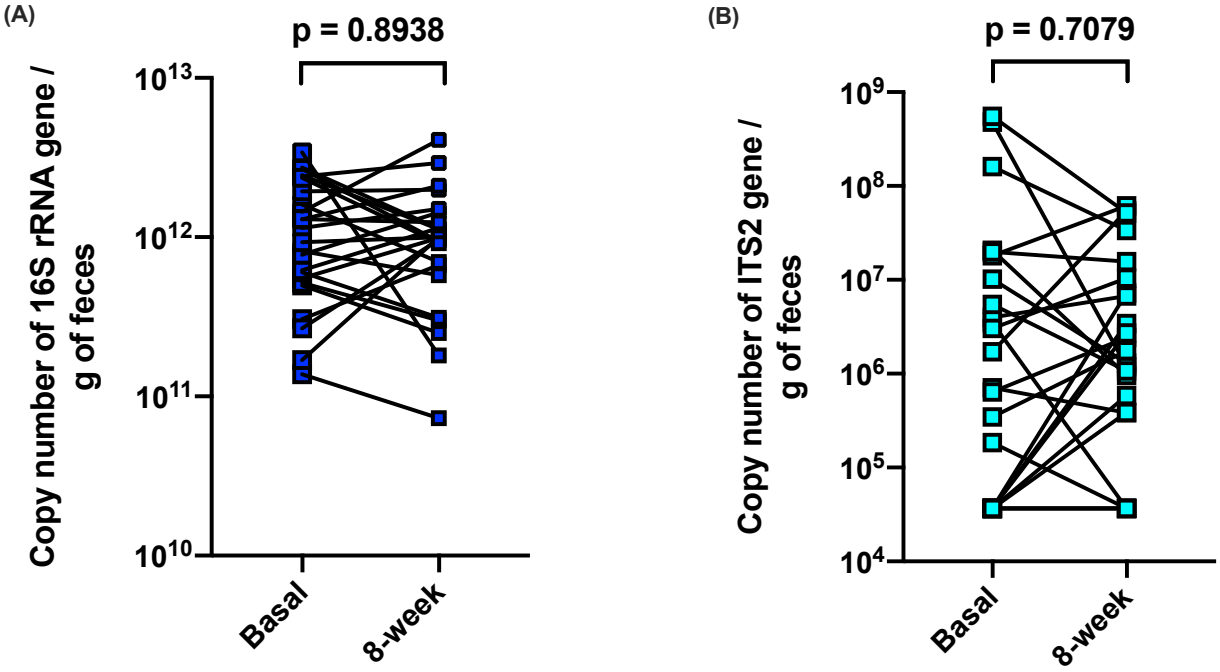

Figure 24. Fungal and bacterial load variations in two time-points. Bacterial load (A) did not show significant variations at basal compared to 8-week samples. Likewise, fungal load (B) showed no significant changes.

Additionally, as seen in Figure 27, the fold changes that reflect the stability over time showed a trend for a more significant variation in the quantity of the ITS2 gene than the 16srRNA gene, but this did not reach statistical significance.

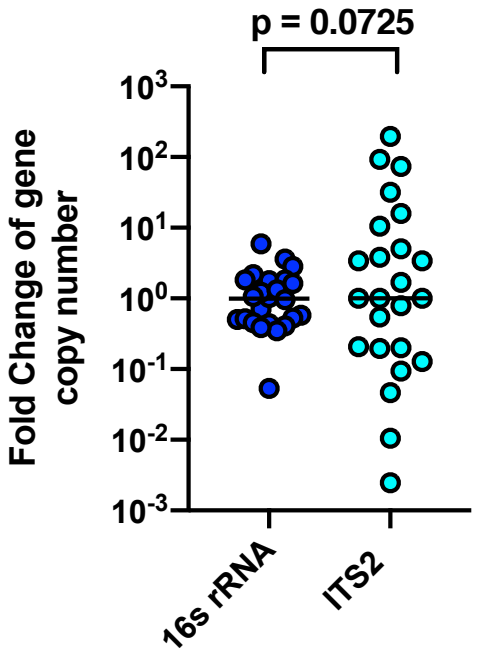

Figure 25. Stability of fungal and bacterial loads. There were no significant differences in fold change between bacterial and fungal loads in healthy individuals.

We ran a Spearman's correlation test to assess the relationship between the copy number of the ITS2 gene and the 16srRNA gene. As shown in figure 28, we found a moderate positive correlation between the fungal and bacterial loads (r_s: 0.50, p = 0.011)

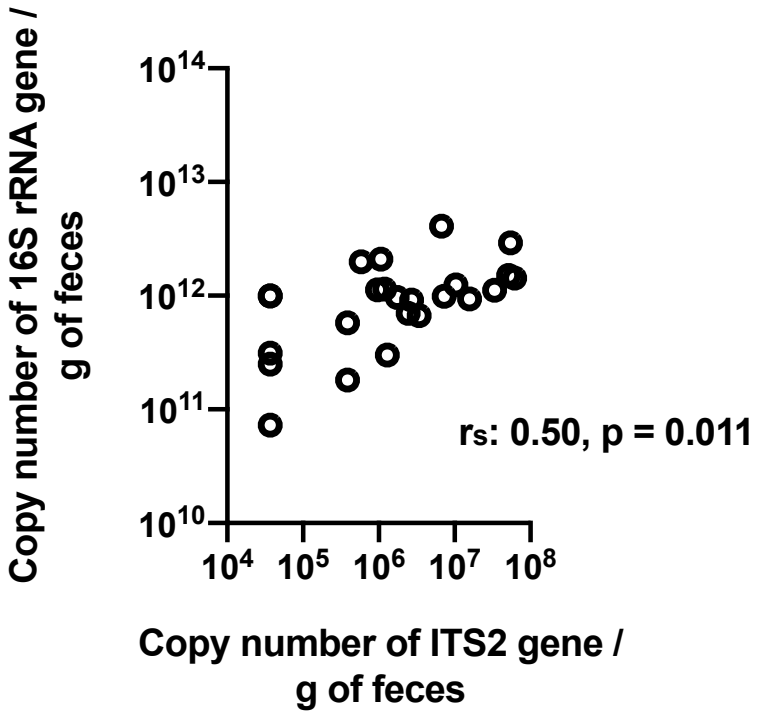

Figure 26. Correlation between Fungal and Bacterial loads in healthy individuals. We found a moderate positive correlation between the ITS2 gene abundance and the bacterial abundance of healthy subjects (r_s = 0.50, p = 0.011)

5.6.2. Characterization of fungal and bacterial load in Ulcerative colitis patients

In UC patients the number of copies of 16s rRNA gene (Figure 29A) was similar among the different groups with a median of 3.88E+11 copies/g of feces [Q1-Q3: 1.92E+11-6.185E+11] for UC long remission, median 5.29E+11 copies/g of feces [Q1-Q3: 1.94E+11-9.64E+11] for UC short remission and median 4.315E+11 copies/g of feces [Q1-Q3: 3925E+11-8.58E+11] for UC flare patients (FDR p = 0.6585).

In contrast, copies of the ITS2 gene were increased in UC flare patients (median 1.95E+06 copies/g of feces [Q1-Q3: 8.10E+04-1.28E+07]) when compared to UC long remission (median 3.68E+04 copies/g of feces [Q1-Q3: 3.68E+04-1.40E+06]) (FDR p =

0.0036) but not significantly different to UC short remission patients (median 5.24E+05 copies/g of feces [Q1-Q3: 3.68E+04-1.66E+06]) (FDR p = 0.2081) (Figure 29B).

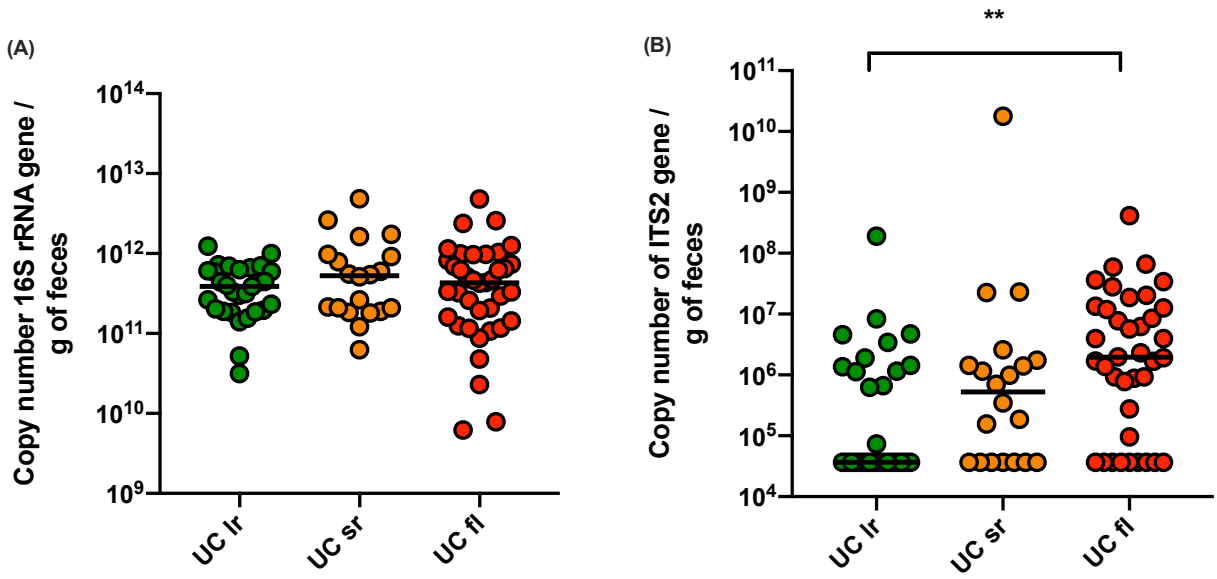

Figure 27. Fungal and bacterial loads in Ulcerative colitis patients. 16S rRNA abundance (A) was similar across UC long remission (UC lr), UC short remission (UC sr), and UC flare patients (UC fl). ITS2 gene copies (B) were significantly lower in UC lr patients when compared to UC fl, but similar to UC sr. **p<0.01

Furthermore, analysis of the ITS2/16S rRNA ratio assessing the frequency of fungi compared to bacteria, showed that this ratio was higher in UC flare patients (median 6.94E-06 [Q1-Q3: 7.29E-07 - 1.92E-05]) when compared to UC long remission (median 2.63E-07 [Q1-Q3: 8.91E-08 - 4.83E-06], FDR p = 0.0005) and UC short remission patients (median 5.60E-07 [Q1-Q3: 1.96E-07 - 3.72E-06], FDR p = 0.0072), while there were no differences between UC long remission and UC short remission patients. (Figure 30).

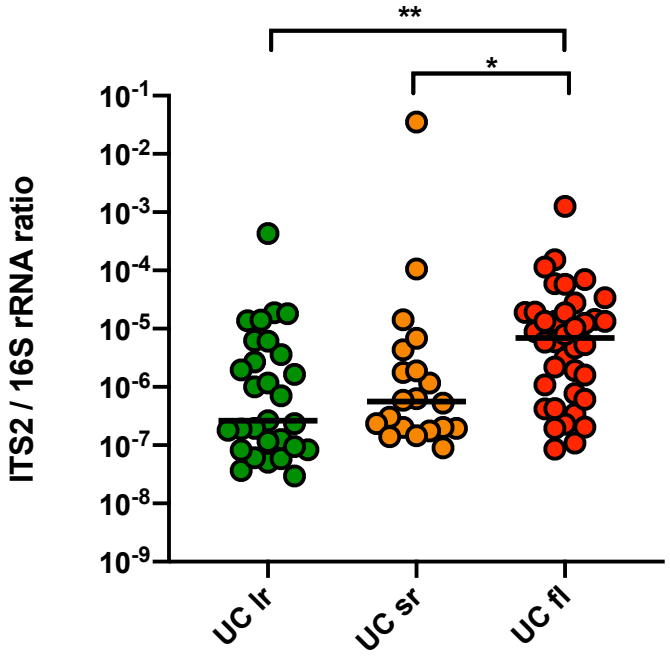

Figure 28. ITS2/16S rRNA ratio in Ulcerative colitis patients. UC long remission patients (UC lr) and UC short remission patients (UC sr) had a lower ITS2/16S rRNA ratio than UC flare patients (UC fl). *p<0.05, **p<0.01.

5.6.3. Fungal and bacterial load differences between UC patients' groups and healthy controls

We compared the bacterial load between UC patients and healthy individuals. UC patients had a decreased quantity of 16srRNA gene copies (median 4.28E+11 copies/g of feces [Q1-Q3: 1.89E+11-7.01E+11]) when compared to healthy controls (median 9.93E+11 copies/g of feces [Q1-Q3: 6.00E+11-1.38E+12], p = 0.0002) (Figure 31A).

However, as shown in Figure 31B, median ITS2 gene copies did not significantly differ between healthy individuals (median 1.53E+06 copies/g of feces [Q1-Q3: 3.85E+05 -

9.61E+06]) when compared to UC patients (median 9.27E+05 copies/g of feces [Q1-Q3: 3.68E+04 - 4.56E+06], p = 0.1593)

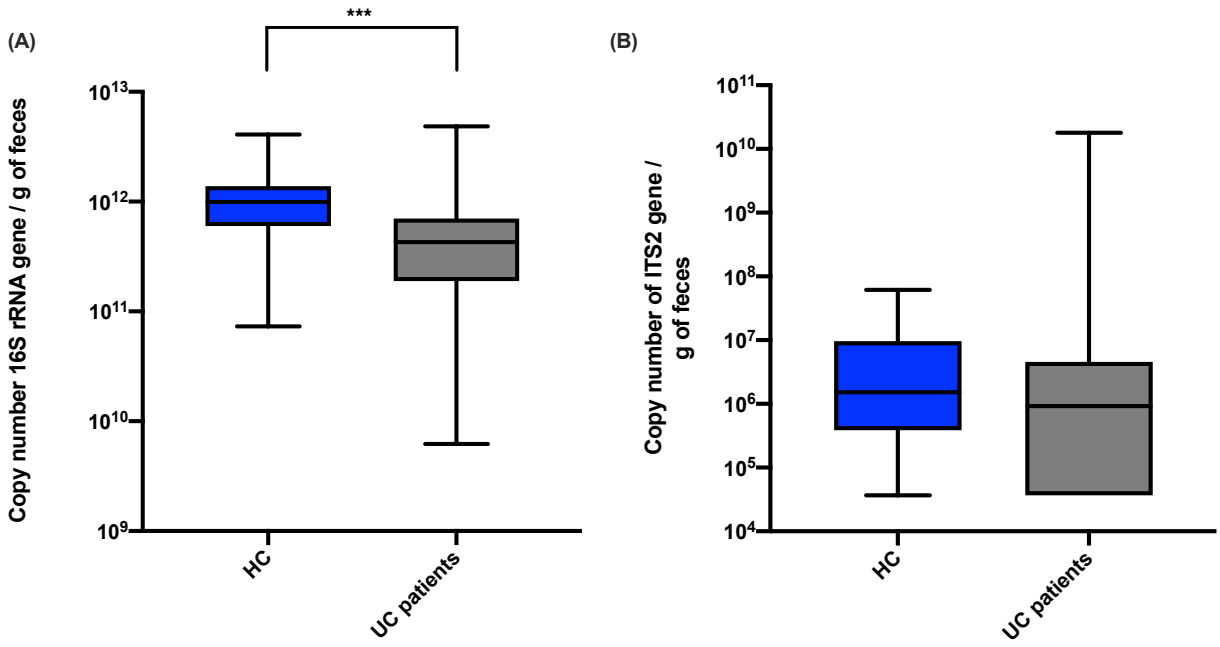

Figure 29. Fungal and bacterial loads in Healthy subjects and UC patients. 16srRNA (A) gene abundance was significantly higher in healthy controls (HC) and UC patients. ITS2 (B) gene copies were similar between HC and UC patients. ***p<0.001

Nevertheless, when comparing UC patients in long remission and healthy controls (Figure 32A), median ITS2 gene copies were decreased in UC long remission (median 3.68E+04 copies/g of feces [Q1-Q3: 3.68E+04-1.40E+06]) when compared to healthy subjects (median 1.53E+06 copies/g of feces [Q1-Q3: 3.85E+05-9.61E+06]) (p = 0.0090). However, the ITS2/16srRNA ratio was not significantly different between healthy individuals and UC long remission patients. (Figure 32B).

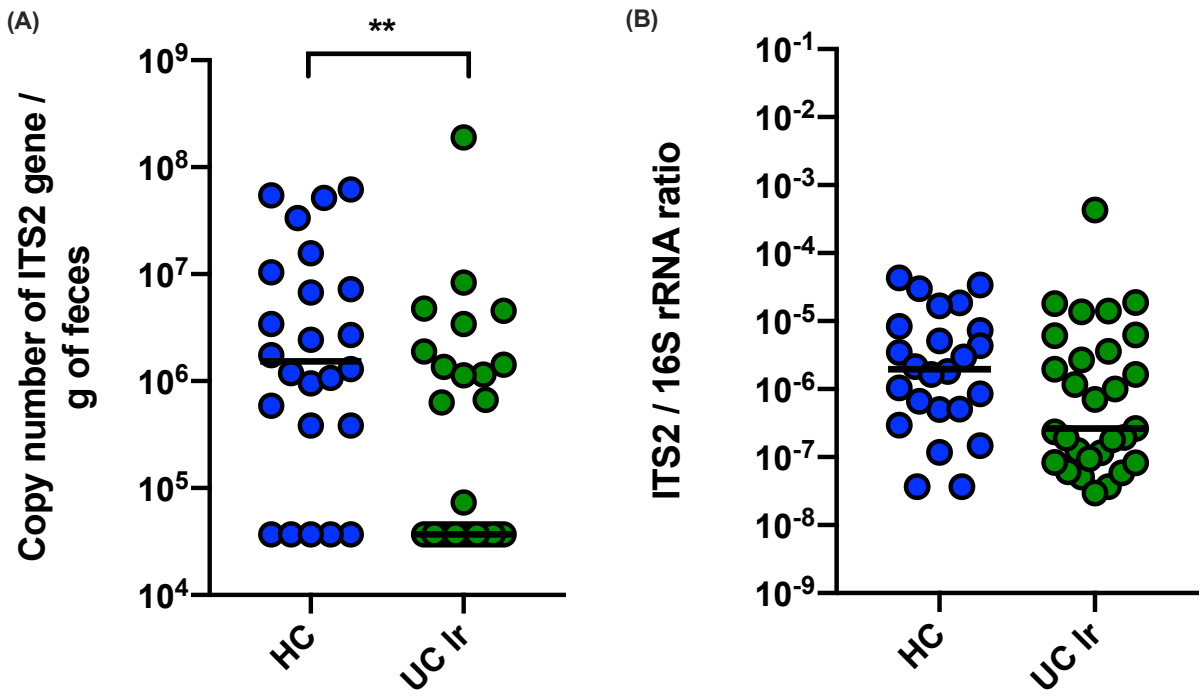

Figure 30. Comparison of ITS2 copies and ITS2/16S rRNA between healthy subjects and UC long remission patients. Fungal load (A) was significantly lower in UC long remission patients (UC lr) when compared to healthy subjects (HC). ITS2/16S rRNA ratio (B) was similar between HC and UC lr. **p<0.01

The etiology of UC involves an aberrant immune response to an imbalanced gut microbiota in genetically susceptible individuals, following unknown triggering events. Alterations in the composition and diversity of the gut microbiota have shown to play a crucial role in the onset and perpetuation of IBD. This gut dysbiosis has been mostly studied and characterized in CD, while studies are scarce in UC.

Clinical, endoscopic, and histological remission positively impacts on the natural history of UC, probably in the direction of true disease modification.

The main objective of this thesis was to study the gut microbiota in UC patients in clinical, endoscopic, and histological remission of the disease long-term and compare it to other UC patients (short remission and active disease) and healthy controls.

We hypothesized that if UC patients are able to achieve these endpoints long-term, it would also be reflected in their gut microbiota.

6.1. Characterization of bacterial microbiota in UC patients in remission and flare

Our study found changes in gut microbiota composition in fecal samples from UC patients, confirming and complementing previous studies. We confirmed dysbiosis in UC patients' samples, but we found differences between the different UC patients' groups and healthy subjects' fecal samples.

Studies have shown that dysbiosis correlates with active disease in UC (169, 234). Therefore, we evaluated if UC patients who reach long-term stable remission present a microbiome closer to so-called eubiosis than patients in shorter remission lengths or active disease. Our results showed that long-remission UC patients' fecal microbiota closely resembles that of healthy controls as reflected by similar diversity, richness, and evenness scores compared to active and short-remission UC patients. UC patients with

short-term remission and patients with active disease presented a significantly lower microbial richness as measured by the number of observed species-level OTUs than in UC patients in long, stable remission and healthy controls.

Furthermore, we found that alpha diversity was lower in UC short remission and UC flare patients than UC long remission and healthy subjects. Microbiome studies have been consistent in finding reduced diversity in IBD patients compared with healthy controls. A lack of microbial diversity seems to be a general theme in several diseases, suggesting that a species-rich ecosystem is more robust against environmental influences. Consequently, diversity seems to be a good indicator of a 'healthy gut'.

When analyzing the similarity in gut microbiota composition between samples (beta diversity), all UC patients, including the long remission group, differed from healthy individuals. Nevertheless, the most significant differences were seen in the short-remission and flare UC patients.

These results imply that the gut microbiota's composition might be permanently different in UC. A possible explanation for this finding is that these subtle differences might precede disease debut and are further exaggerated when inflammation occurs. UC patients who achieve stable long-term remission have less striking differences in their microbiota composition than healthy subjects.

We identified specific taxa that were associated with healthy individuals and long-remission UC at different taxonomic levels. Most notably, *Akkermansia muciniphila* relative abundance was significantly decreased in active and short-remission UC patients. Our study provided consistent results for the taxonomic lineage from Verrucomicrobia to *Akkermansia. Akkermansia* is the most abundantly identified mucolytic mucosa-associated bacterium. Since its discovery, evidence is accumulating suggesting a beneficial role for *A. muciniphila* and it is associated with a protective or anti-inflammatory role in health, which may be lost in IBD (191, 235). The species has the ability to modulate

host immune responses and may play a role in immune-tolerance to commensal microbes (236).

The role of *A. muciniphila* in modulating human immunological homeostasis is showed in the recent study that showed that *A. muciniphila* induce homeostatic IgG production and antigen-specific T cell responses in mice (237) and that *A. muciniphila* treatment ameliorated Dextran Sulfate Sodium (DSS)-induced colitis in mice (238).

Significant reductions in *A. muciniphila* have been demonstrated in both fecal samples and mucosal biopsies of patients with UC (191, 239-241). Germ free mice colonized with *A. muciniphila* do not develop microscopically visible inflammation, strengthening the argument for a protective role of this microbe in the setting of UC (236). A recent study quantified *A. muciniphila* within the mucus gel layer in UC patients with active and quiescent disease (242). This study found an inverse relationship between *A. muciniphila* and inflammation, with higher inflammatory scores associated with a reduced abundance of *A. muciniphila*. Furthermore, a study of FMT in UC patients found that *A. muciniphila* in donor samples was predictive of a beneficial response to fecal microbiota transplantation in UC (193).

A. muciniphila produces the short-chain fatty acids propionate and acetate, but also succinate as one of the major metabolites from mucin degradation. Succinate is an organic anion; it is one of the end products of dietary fiber consumption, like short-chain fatty acids, and is an important microbial product for beneficial metabolic effects (243, 244). Members of the Prevotellaceae family, such as the genus *Prevotella* and *Paraprevotella*, also produce succinate. We found that healthy subjects and UC long remission patients had a higher relative abundance of genus *Paraprevotella* than UC short remission and UC flare patients.

The depletion of SCFA-producing bacteria has consistently been found in samples from Crohn's disease patients, whereas studies focusing on UC patients have yielded conflicting results. Reduced abundances of butyrate producers such as *Faecalibacterium*

prausnitzii and *Roseburia intestinalis* in IBD are indicators of unfavorable disease evolution. The fecal content of SCFAs is also lower in IBD patients than in healthy controls (245). Although *Faecalibacterium* and *Roseburia* showed a similar abundance among all groups that we studied, we observed a trend toward a lower abundance of *F. prausnitzii* in UC short remission and flare patients than UC long remission and healthy subjects.

Interestingly we found *Coprococcus* abundance to be decreased in all UC patients, including long remission UC patients when compared to healthy controls. *Coprococcus* is among the essential butyrate-producing genera in the human gut microbiota. These bacteria promote colonic health by mediating anti-inflammatory and antitumor effects, as well as providing energy for colonocytes (246). A low presence of *Coprococcus* has been linked to IBD and lower treatment response (189).

Several unclassified species of *Ruminococcus* and *Lachnospiraceae* (*Clostridium* cluster IV and XIVa) showed a higher abundance in healthy controls and UC long remission than in UC short remission and flare patients. Surprisingly, *Blautia* and *Dorea,* which also belong to the Lachnospiraceae family, were significantly higher in UC short remission patients than in all other subjects.

Clostridium species are one of the richest bacterial clusters and mainly composed of *Clostridium* cluster IV and XIVa. Several species within this genus are strongly involved in maintaining overall gut function by releasing butyrate that is essential as fuel for colonocytes. Decreases in *Clostridium* species have been associated with loss of gut microbiome colonization resistance (reduced diversity and community stability over time) in subjects who receive broad-spectrum antibiotics (247).

Cocktails of *Clostridium* species belonging to clusters XIVa, IV, and XVIII promote anti-inflammatory immune responses by activating regulatory T cells in animal models (248), as well as a study that shows that inoculation of clostridia during early life reared mice resistant to colitis (249).

Underrepresentation of these taxa in UC patients has been observed in other studies (194, 250). Furthermore, two FMT trials in UC patients reported that several taxa in the donor were associated with FMT response, among these *Ruminococcus* and *Lachnospiraceae* (71, 72). Our findings further support the notion that *Clostridium* clusters IV and XIVa are consistently associated with remission.

Finally, *Alistipes* abundance was significantly higher in UC long remission patients than the other groups, including healthy subjects. *Alistipes* is a relatively recent sub-branch genus of the Bacteroidetes phylum (251). There is contrasting evidence indicating that *Alistipes* may have protective effects against some diseases, including colitis, while other studies suggest that *Alistipes* is pathogenic in colorectal cancer. It has been suggested that *A. finegoldii* may be a protective species against colitis (252).

We also identified bacteria with a higher abundance in UC flare patients than in healthy controls and UC long remission patients. *Collinsella* was more abundant in UC flare patients than healthy controls and UC long and short remission patients. A study in a humanized mouse model of rheumatoid arthritis reported that *Collinsella* abundance correlated with increased gut permeability (253). A study that sought to find a microbial signature to distinguish between CD and non-CD showed that *Collinsella,* which was found mostly in UC cases, allowed for the discrimination between UC and CD (155). In contrast, a prospective pediatric IBD cohort drawn from 28 referral centers across the USA reported that the presence of increased *Collinsella* in stool samples was associated with a fistulizing phenotype of CD (254).

Peptostreptococcus anaerobius abundance was also found to be higher in UC flare patients than in all other groups. Species of the genus *Peptostreptococcus* are found as commensals in virtually every type of mucosa that lines humans. They have pathogenic potential when they are traumatically introduced deep into tissues, or when the host becomes weakened.

Furrie and colleagues described their simultaneous quantitative analysis of immune responsiveness to over 35 intestinal bacterial isolates in IBD subjects (255). Significantly higher systemic antibody responses were mounted in UC towards *Peptostreptococcus anaerobius*, parallel with higher recovery rates of this strain from the colonic mucosae. *P. anaerobius* has been observed to be enriched in samples of colorectal cancer patients, and its oncogenic potential has been described in mice (256, 257).

Furthermore, a study of newly diagnosed CD patients found that *Peptostreptococcus* was one of the genera positively correlated with the severity of the disease (258). In contrast, a recent study showed that *Peptostreptococcus russellii* could colonize the mucus layer and mediate an increase in goblet cell number and reduce susceptibility to DSS-induced colitis in mice, likely by producing the tryptophan metabolite indoleacrylic acid (259).

We found that *Streptococcus* abundance was higher in UC short remission and UC flare patients than in UC long remission and healthy controls. Although many strains of *Streptococcus* are non-pathogenic and occur as commensal flora, some species of *Streptococcus* are responsible for numerous diseases. Association of *Streptococcus bovis* with IBD and colon cancer has been reported (260), and increases in abundance of *Streptococcus spp* have been found in stool samples of UC patients when compared to healthy controls (250, 261).

To complement the 16S rRNA gene amplicon sequencing data, we also determined specific species associated with UC in other studies with qPCR. Our results mimic those of the 16S rRNA gene data, further validating these findings.

6.2. Gut microbiota in UC and prediction of disease activity

UC long remission and UC short remission patients were followed for an additional 24 months after inclusion to determine the disease's activity. We examined the association of patients' gut microbiota data and the occurrence of a flare. The presence of

Akkermansia muciniphila and higher diversity (by Shannon index) were protective factors for flare development. Furthermore, the presence of *A. muciniphila* at inclusion was predictive of remission at two years in UC patients.

Taken collectively, these findings are consistent with previous studies in that the gut microbiota of UC patients presents a gain of harmful microbes and the loss of beneficial ones. Still, our findings prove that UC patients who can achieve long-term deep and histological remission indeed present a gut microbiota closer to health than the active and short-remission groups, and thus less dysbiotic.

The dysbiosis seen in UC patients is evident in the UC flare samples and, to a less extent, in UC short remission. Beneficial species such as *A. muciniphila* and several butyrate-producing species were reduced in UC flare and short remission but showed similar abundance in UC long remission patients and healthy subjects. Furthermore, potentially harmful species were more abundant in UC flare samples than in UC patients in short and long remission. Nevertheless, patients in remission for a shorter length of time also show dysbiosis. Dysbiosis described in this study seems to be associated with differences in inflammation, as observed in previous studies. More substantial alterations of gut microbial composition in UC could be explained by more severe degrees of inflammation. However, some gut microbiota characteristics and specific taxa were still different in the gut microbiota of UC patients in long remission compared to healthy subjects.

A 'healthier' gut microbiota might be the ultimate goal to achieve in UC patients. Like clinical, endoscopic, and histologic remission, it may be associated with improved short-term and long-term outcomes. Some aspects of the gut microbiota might even be able to predict flare-free outcomes, such as a higher diversity and the presence of specific bacteria, such as *A. muciniphila.*

6.3. Characterization of fungal and bacterial loads in the studied groups

Additionally, we determined the fecal fungal and bacterial loads in UC patients and healthy controls to explore the microbial composition further.

We analyzed and compared the fungal and bacterial load by absolute quantification using qPCR. A recent study compared the fungal and bacterial composition in healthy individuals, by ITS2 and 16srRNA gene amplification and sequencing. Using the number of observed OTUs and the Shannon index, they observed a significantly lower fungi diversity than bacteria (132).

In addition to their observations, by targeting the same DNA sequences, we have shown that healthy individuals have a significantly lower fungal load than bacterial load and that these two parameters showed a positive moderate correlation.

A parameter that distinguishes the fungal from the bacterial microbiota in healthy individuals is its stability across time (202). We found that, compared to the bacterial microbiota, the composition of the fungal microbiota appeared to be less stable, suggesting that this fraction of the microbiota is composed of more transient microbes that may be more influenced by environmental signals.

In fecal samples from UC patients, we observed differences in the fungal microbiota between the different groups. The fungal load was increased in UC flare patients than in UC long remission, while there were no differences in the bacterial load. This high number of fungi could be involved in the inflammatory response of UC patients.

The fungal/bacterial load ratio was also higher in UC flare patients than UC long remission and UC short remission patients. These findings suggest that changes during inflammation might affect fungi and bacteria differently and induce an altered inter-kingdom relationship in UC.

When comparing healthy controls and UC patients, we observed that the fungal load was lower in UC long remission patients, yet the fungal/bacterial load ratio was not significantly different.

A recent study (262) investigated the intestinal mycobiota dynamics in UC patients undergoing FMT (72), from the FOCUS study. The authors targeted the ITS1 region and found that high Candida abundance before FMT in UC patients was associated with clinical response to FMT and decreased post-FMT Candida abundance. These results suggest that reducing certain fungi results in ameliorated disease severity.

Taken together, these data strongly suggest a link between fungi number and inflammatory status in UC. However, more studies need to be performed to determine if the fungi control the inflammatory status or if remission induced by treatment favors changes in the mycobiome.

6.4. Limitations and strengths

There are some limitations of our study. First, we evaluated the microbiota composition only in fecal samples. Fecal samples might not entirely reflect the microbiota dysbiosis as disease-relevant, and more adherent bacteria are probably ‘diluted’ in feces. Although our rationale to use fecal samples is the ease of collection for the patient, the non-invasive nature of the procedure and feasibility for repeated sampling instead of mucosal sampling.

Our study's cross-sectional design also poses significant limitations, as longitudinal studies allow for a more robust association of changes in the microbiota of patients by accounting for time-point variations in the microbiota composition and determining causality. Lastly, we did not collect dietary data from our patients, and since diet is a well-

known factor that influences the gut microbiota, it may be a non-controlled confounder in this study.

Our study's strengths include the number of subjects recruited and samples analyzed, complete clinical and demographic data, and our follow-up sub-cohort. We also studied the microbiota beyond the bacterial component, to include the fungal microbiota. We chose to include patients with left-sided or extensive disease as these patients tend to have a more complicated disease course than proctitis patients (263).

We selected patients in extreme spectrums of the disease, as we included active disease patients, patients with recent activity, and patients with extended periods of inactivity to compare with healthy individuals. Finally, we performed two different methods to evaluate the reproducibility of some of our microbiota data.

To our knowledge, this is the first study to evaluate the microbiota composition in UC patients with a long, stable, flare-free disease. UC patients who can achieve long-term deep remission of their disease present a gut bacterial microbiota closer to health. Whether these findings represent a true disease modification or are the consequence of adequate control of disease activity remains to be evaluated in large, longitudinal studies. Furthermore, some characteristics and specific taxa will probably never be recovered in the gut microbiota of UC patients in remission; we believe that a microbial endpoint might be a realistic goal in future clinical studies. Some aspects of the gut microbiota might predict flare-free outcomes, such as a higher diversity and the presence of specific bacteria, like *A. muciniphila*.

Finally, in this study we explored the fungal microbiota. Our findings raise new and interesting questions that should be addressed in larger cohorts of UC patients.

CONCLUSIONS

1. There is a change in the normal gut microbiota in Ulcerative colitis patients, with a reduction in diversity and richness, underrepresentation of beneficial bacteria such as *Akkermansia muciniphila*, several butyrate-producing bacteria, and the gain of potentially harmful microbes. This dysbiosis is greater in UC patients in disease-flare but is also present in UC patients in short remission, albeit to a lesser extent.

2. Ulcerative colitis patients who are able to achieve a long-term, stable, deep, and histological remission of their disease present a gut bacterial composition that is closer to health, and thus less dysbiotic. However, some characteristics and specific taxa, like *Coproccocus* might be permanently lost in UC patients.

3. Dysbiosis described in this study is associated with differences in inflammation with substantial alterations of gut microbial composition associated with more severe degrees of inflammation.

4. A 'healthier' gut microbiota might be the ultimate goal to achieve in UC patients. Like clinical, endoscopic, and histologic remission, it may be associated with improved short-term and long-term outcomes. Some aspects of the gut microbiota might even be able to predict flare-free outcomes, such as a higher diversity and the presence of specific bacteria, like *Akkermansia muciniphila*.

5. Beyond the bacterial component, there is a link between fungi abundance and inflammatory status in UC. Patients in disease-flare present a greater abundance of the fungal load than patients in remission, but these results must be interpreted cautiously and further evaluated in larger, longitudinal studies.

FUTURE PERSPECTIVES

Our study about the characterization of the gut microbiota in ulcerative colitis in remission raises new and interesting questions.

First, we proved that the gut microbiota in UC patients in long remission presents a more 'eubiotic' microbiota composition than their flare and short remission counterparts. It remains to be seen if this represents a true disease modification or the consequence of adequate control of disease activity.

Secondly, the changes that we found in fungi abundance and fungal to bacterial load ratio related to UC disease activity or remission should be further characterized in large, longitudinal cohorts. To obtain a holistic picture of the intestinal ecosystem in a disease such as UC, microbiome studies should go beyond the bacteria to include the other constituents of the intestinal microbiota.

Finally, we believe that achieving a long-term deep remission should include a consideration for the gut microbiota. Microbiota changes play an important role in UC, and researchers may focus on finding a way to restore microbial composition on UC. A microbial endpoint might be a realistic goal in future clinical studies.

Recovery of the gut microbial ecosystem may potentially be useful for disease monitoring and to assist in medical decisions such as reduction of intense immunosuppression therapies.

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